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ANTONIE VAN LEEUWENHOEK

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THE CENTRAALBUREAU VOOR SCHIMMELCULTURES

HALF A CENTURY'S EXPERIENCE WITH MOULD CULTURES

by

AGATHE L. VAN BEVERWIJK

(Received October 30, 1958).

History.

The desirability to establish a collection of pure cultures of fungi available for scientific research was discussed at the meeting of the Association Internationale des Botanistes at Leiden in 1903. Three years later the Centralstelle für Pilzkulturen was founded and housed at the botanical laboratory at Utrecht under the directorship of Professor Dr F. A. F. C. Went. In 1907 Professor Went turned over the collection to Dr Johanna Westerdijk who was then already director of the phytopathological laboratory "Willie Commelin Scholten". The collection at that time contained nearly eighty cultures, a list of the fungus names, printed in 1906 in the Botanisches Centralblatt, was reproduced for distribution as the first catalogue from which cultures could be ordered. The name of the collection was changed to Centraalbureau voor Schimmelcultures (C.B.S.). The C.B.S. now has nearly 10.000 strains of about 6.300 different species of filamentous fungi, yeasts and Actinomycetes. The first and the latest (1957) lists of cultures are shown on the table in fig. 1.

Since 1922 the yeast division of the C.B.S. is housed at the Laboratory of Microbiology at Delft. It was under the supervision of Professor Dr A. J. Kluyver until his death in 1956. He was succeeded by Professor T. O. Wikén. The aim of the Centraalbureau voor Schimmelcultures is to maintain a large collection of cultivable fungi and to distribute cultures to research workers in science and

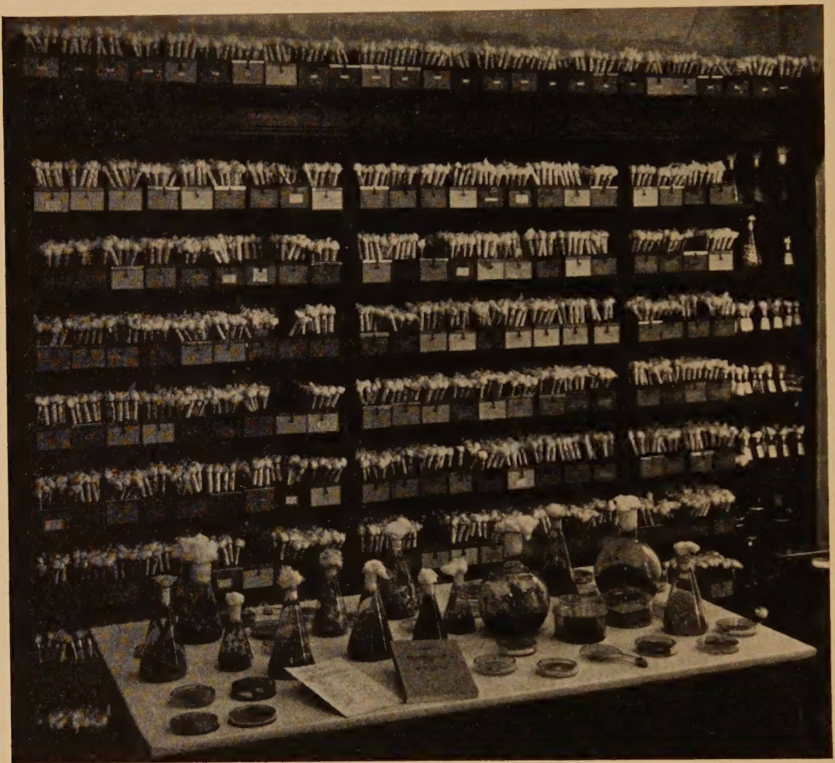

Fig. 1. On the table a permanent exhibition of fungi, with the first and the latest catalogues. In the background a fractional portion of the stock of cultures, growing in cotton plugged tubes.

industry all over the world. Moreover cultures can be sent to the C.B.S. for identification; industrial and scientific research workers can ask for advice in their problems with moulds. New specimens are obtained through exchange and on request. Many research workers deposit their new species or important strains with the C.B.S. collection, where these cultures remain available for further research.

Fungi, especially yeasts, were used and even cultured long before people knew they were doing so. At a very early period the processes of baking and brewing were practised. A piece of dough from each baking lot was carefully preserved to provide the leaven for the next.

Alcohol was produced from different substrates by methods independently discovered in various parts of the world. In mediterranian countries wine was obtained from grapes, in the colder northern territories barley was used as raw material for the production of beer, inhabitants of the Orient made their alcoholic beverages starting from rice and sago. It took many centuries, however, before these processes, crudely, but effectively handled, became subject of scientific investigation.

ANTONIE VAN LEEUWENHOEK (1632–1723) was one of the first to discover the existence of micro-organisms. He noticed that yeast consists of minute spherical particles which give off bubbles of gas in fermenting wort.

The first attempt to culture moulds was made by the Italian botanist PIER' ANTONIO MICHELI (1679–1737). On freshly cut pieces of melon he smeared spores ("seeds" as he called them), of *Mucor* on one side and spores of *Aspergillus* on the other side. He noticed that each side produced its own kind of fungus. Then he sowed *Botrytis* spores on two pieces of melon, kept one of them under glass, leaving the other exposed. On the covered piece only *Botrytis* developed. On the exposed piece "*Mucor* was revealed with black and shining heads, no one having planted it, but the seeds having fallen there by chance" (Nova Plantarum Genera (1729) Observatio V, cited from the English translation in BULLER, 1915, p. 22). In this way MICHELI showed that spores of moulds are distributed through the air.

It was, however, more than a century later that the ingenuous experiments of LOUIS PASTEUR (1822–1895) convincingly proved that living micro-organisms floating about in the air, were the cause of infusions "turning bad". He definitely proved that moulds and bacteria were independent organisms not originating from dead organic material. The investigations of PASTEUR opened up a new branch of science: fermentation through micro-organisms.

Since MICHELI's first attempts, culture techniques have greatly improved. OSCAR BREFELD (Germany 1839–1925) in his "Untersuchungen aus dem Gesamtgebied der Mykologie" gives many detailed accounts of his observations of spore germination, growth and development of fungi. He saw the importance of growing pure cultures on sterilized culture media using gelatine to make his media solid.

To be admitted into the world of science an organism must have a "scientific" name. Mycology is a comparatively young specialism of botany and until the end of last century it was still in its purely taxonomic period. Great mycologists: CHRISTIAN HENDRIK PERSOON (South-Africa, Germany, France, 1761–1836), LEWIS DAVID VON SCHWEINITZ (USA, 1780–1834), ELIAS MAGNUS FRIES (Sweden, 1794–1878), CORDA (Prague, 1800–1849), the TULASNE brothers (France, 1815–1885), C. A. J. A. OUDEMANS (Netherlands, 1826–1906), HEINRICH ANTON DE BARY, "the founder of modern mycology", (Germany, 1831–1888), PIER' ANDREA SACCARDO (Italy, 1845–1920), CARLOS LUIS SPEGAZZINI (Argentina, 1858–1926) did much of the basic work in collecting and classifying fungi. The material which they studied and described is partly preserved in herbaria. PERSOON's herbarium for instance, is kept at the Rijksherbarium at Leiden. Valuable type specimens thus remain available for comparison to later scientists dealing with problems of classification.

Increased knowledge of fungi and their modes of living widened the interest in these organisms. Fungi were detected as parasites on living plants, causing serious losses in crops. The early plant-pathologists were almost entirely concerned with fungus diseases and modern phytopathologists too, must have a sound knowledge of mycology.

With the intensified industrialization and the prolonged storage of raw materials and ready products, the spoilage caused by mouldy deterioration became a factor of great economic importance. Hence chemical industries became interested in the production of substances for controlling mould growth. In recent years the discovery of the biochemical activities of moulds, the production of various organic acids, of vitamins, enzymes and above all of antibiotics has brought the fungi into the field of industry. The industries concerned are entirely dependent on cultured fungi that are well identified and true to species. That is why ultimately they must rely on the work of the taxonomists. Systematic mycology, from a historical point of view the basis of mould science, remains of fundamental importance for all further research concerning fungi.

AIMS OF THE CENTRAAL BUREAU VOOR SCHIMMELCULTURES.

The C.B.S. aims at maintaining a large collection of living fungi to supply diverse fields of science and industry with the required cultures, identifications and advice.

It is not always possible exactly to detect for what purpose the cultures are requested. Nor is it possible to draw a rigid line of demarcation between scientific and industrial research. An investigation which is set up on a purely scientific basis, may suddenly become of great industrial moment. On the other hand researches, carried out at industrial laboratories may give valuable contributions to science.

For the sake of surveyability, however, we shall divide the various approaches to mycological research into the following subjects and describe in brief what contributions the C.B.S. can give to these fields of investigation.

1. scientific mycological research.
2. phytopathological, agricultural and similar laboratories.
3. medical mycology.
4. industrial mycology.

1. Scientific research.

a. Morphology and taxonomy.

What is termed pure mycology concerns the study of morphological structures and modes of development of fungi with a view to their classification. The material studied can be either the living fungus in its natural habitat, or an isolation of it in pure culture, or a dried herbarium specimen. There always is some controversy between herbarium mycologists and culture mycologists. The latter, when handling dried material, often feel at a loss because of the sparseness of a valuable specimen, whereas the herbarium specialist looks rather suspiciously at the contents of a culture tube. As to the best method of approach, much depends on the kind of fungus studied. The larger fungi, the toadstools and mushrooms which but rarely develop fruitbodies in culture, can best be studied in their natural habitat or in dried condition, whereas for the taxonomy of yeasts, of most of the Phycomycetes, for the study of life cycles, especially of heterothallic fungi, pure cultures are essential. A herbarium specimen is often only a snapshot out of the life history of a fungus. Cultures, however, allow of the study of the interrelationship between the perfect and imperfect stages, and can prove that they are states of the same organism. The variability of a fungus can best be studied in pure culture, as this method permits one to start from a single spore and follow its development under different culture conditions. By this method SNYDER and HANSEN (1940, 1941, 1945), studied the variability of the species of the genus *Fusarium*. So for the classification of fungi, cultures and herbarium specimens are equally important, either method having its potentialities and limitations.

The culture collection of the C.B.S. has in course of time given many contributions to taxonomic research. Frequently one or more species of a genus are requested for a comparative study. Sometimes all the species of a genus are required by a specialist monographing that particular genus.

ZYCHA (1935) when preparing his book "*Mucorinae*" examined the species of this group then present in the C.B.S. collection. More recently, in 1946, transfers of all the species of the genus *Penicillium* were sent to the Northern Regional Research Laboratory of the United States Department of Agriculture at Peoria, Illinois, where RAPER and THOM (1949) were then preparing their monograph "The Penicillia". HESSELTINE examines cultures of the C.B.S. for his revision of different genera of the *Mucorales*. Some years ago all the species and strains of *Paecilomyces*, *Spicaria* and *Isaria* were sent to BROWN and G. SMITH (1957) for their monograph on *Paecilomyces* and *Byssochlamys*. At present, cultures of *Sporotrichum* and allied species are being studied for a revision of that genus. The collection also inspires the mycologists of the C.B.S. to taxonomic publications:
VAN BEYMA THOE KINGMA, who was on the staff from 1927 till 1944, wrote a number of papers on taxonomic subjects during those years. LODDER and KREGER-VAN RIJ (1952) monographed the yeasts, present in the collection. DE VRIES (1952) studied a number of species of the genus *Cladosporium* for his doctor's thesis. SCHOL-SCHWARZ is revising the genus *Epicoccum*. Several species of *Aspergillus*, *Penicillium* and *Eurotiaceae*, which were sent to the C.B.S. for identification, but proved to be new to science, were described by STOLK (1954, 1955). New species of *Helicosporae* were isolated and described by VAN BEVERWIJK (1953, 1954).

Guest scientists regularly come to study a particular group of fungi at the C.B.S. These visits of specialists are obviously most important for the culture collection. The cultures of the genus studied are critically examined by a scientist well versed in that group, doubtful and not typical strains are deleted, new isolates are obtained. Moreover the mycologists of the C.B.S. can discuss problems of nomenclature and taxonomy with the specialist. In recent years the genera *Pythium*, *Phytophthora*, *Chaetomium* and several others were thoroughly overhauled.

b. Physiology.

The functions and activities of living fungi may be studied in nature, but as the natural habitat of a fungus is rather complex, the simplified and controlled conditions of the laboratory experiment are more suitable to these investigations. Thus this branch of fungus science is almost entirely dependent on cultivated fungi for its material. The experimenter can obtain his cultures from a reliable culture collection. Yet some knowledge of the morphology of the fungi may save him trouble. MARTIN (1958) mentions a case where experiments on cellulolytic activity, started with *Chaetomium globosum*, gave completely negative results, because the *C. globosum* had been entirely replaced by *Rhizopus stolonifer*, which the experimenter had not noticed, not knowing the difference between the two fungi.

Fungus physiology is a rapidly expanding subject with specializations in many different directions often with a tendency to emphasize the biochemical side of the problem and to drift away from the biological sphere. Whereas the data concerning the physiology of the higher plants are based mainly on experiments with only a few species, a great number of different species of fungi are used in the study of fungus physiology. This is partly because the simple structure of the fungi and their rapid growth make these organisms very suitable for experiments, partly it is due to the fact that fungi show a great diversity in their response to external conditions such as food requirements, light and temperature.

A few examples concerning fungi and vitamins may illustrate this diversity: Many fungi are able to develop normally on a synthetic medium containing no vitamins, e.g. many *Aspergilli* and *Fusaria*, *Rhizopus nigricans*, *Septoria nodorum* proved to be "self-sufficient" with respect to vitamins. Either these moulds do not need vitamins in their metabolism or they are able to synthesize them in sufficient quantities from the compounds of the vitamin free medium in which they are tested. Several fungus species have become of economic importance because they synthesize vitamins as metabolic products:

Eremothecium ashbyii produces riboflavin and is used in the commercial microbiological synthesis of this substance. *Choanephoraceae* produce beta carotene, a precursor of vitamin A. Vitamin B_{12} is synthesized by *Streptomyces olivaceus* and other micro-organisms. A number of fungi again, do not grow unless certain vitamins are present in their culture medium. These "vitamin-deficient" fungi are of importance as test-organisms in vitamin assay. The use of micro-organisms for the estimation of vitamins has many advantages above the animal feeding trials. The results are obtained quickly, the apparatus is comparatively simple and the micro-organisms are usually sensitive to much smaller amounts of vitamins than are animals. Thus thiamin is estimated by measuring the growth of *Phycomyces blakesleeanus*. *Saccharomyces carlsbergensis* is used as a test-organism for pyridoxine and for pantothenic acid. Different mutant-strains of *Neurospora crassa* and *N. sitophila* are used to assay inositol, biotin and p-aminobenzoic acid.

These few instances from one of the many chapters of fungus physiology illustrate that a collection of fungi, having to provide the physiologists with the necessary cultures for their experiments, has to carry many different strains of a great number of different fungi, true to species and in as active a condition as possible. To maintain the biochemical properties of a fungus in culture is a problem of constant consideration for the curator of a culture collection. Lyophilization of the strain, when in optimal condition, may be a safeguard to maintain its metabolic properties. Further investigations into the nutrition of fungi may give valuable indications for the maintenance of fungi on culture media.

2. Phytopathology.

From the very beginning the C.B.S. has been in close contact with phytopathology. For nearly 50 years Professor WESTERDIJK has been director of the C.B.S. as well as of the phytopathological laboratory "Willie Commelin Scholten". During those years the two institutes developed to such an extent that they are now under different directorship but they remain housed in the same building, now extended, much to the profit of either.

Phytopathologists engaged in the study of fungus diseases of plants will need some knowledge of general mycology. They must know where to locate the fungus they observe, whether it is likely to be a saprophyte growing on dead or dying parts of the plant, or whether it is a parasite. In that case an exact identification is essential for the diagnosis of the disease. In many cases it will be necessary to isolate the pathogenic fungus, especially if artificial infection experiments have to be carried out.

The C.B.S. contributes to phytopathology in different ways: by supplying cultures and by carrying out identifications. The cultures of plant pathogens present in the C.B.S. collection are useful material for making phytopathologists familiar with the morphology of the fungi they have to deal with. Industries producing fungicides, order cultures of plant-parasites to carry out preliminary *in vitro* tests with their products. As far as virulence is maintained on agar and natural culture media the cultures can be used for infection experiments. It is not possible for the C.B.S. to check up on the virulence of the cultures. To try this out would mean that this institute should also have to maintain a garden of host plants for all the different pathogens and to inoculate and re-isolate the strains each summer.

To our experience some pathogens e.g. *Helminthosporium gramineum* and *Cercospora beticola* soon lose sporulation and become avirulent in culture. Many other plant parasites can be grown on agar media without losing pathogenicity. *Ascochyta pisi* isolated by WEHLBURG in 1930, still causes leaf spot in peas. A number of strains of *Cladosporium cucumerinum*, one of which was isolated by Professor WESTERDIJK in 1913, are regularly used to test cucumber hybrids on their resistance to gummosis. *Pythium debaryanum* cultivated for over 10 years on agar media still causes damping off of seedlings. Freshly isolated strains are regularly added to the collection, old and unimportant strains are deleted.

As the exact identification of a pathogen, especially in groups such as *Phycomycetes*, *Sphaeropsidales* and the notoriously difficult genus *Fusarium*, often requires the trained specialist, many phytopathologists in the Netherlands and abroad send their isolates to the C.B.S. to be identified.

3. Medical mycology.

Medical mycology is a special branch of fungus science, dealing with highly specialized organisms: moulds and yeasts living on or in man and animals. Cultures are often the only means by which the identity of the pathogenic organisms can be rightly diagnosed. As a rule hospital laboratories are not equipped for mycological techniques and the staff are unacquainted with the methods and the literature used for the identification of the pathogenic fungi. It was therefore considered desirable to have a centre in the Netherlands that could deal with the fungal part of the mycoses. In 1952 it became possible to add this division to the C.B.S. under supervision of a specialized medical mycologist. Cultures of moulds, already isolated at the hospital can be sent in for identification. Mostly, however, material such as scrapings from skin lesions, hairs, nails, sputum or parts of internal organs suspected of mycotic infection are received for isolation and identification of the pathogen. Most of the fungi causing mycoses are cultivable on artificial media, although many dermatophytes soon become irreversibly pleomorphic (sterile) in culture, not losing, however, their pathogenicity. Each year a practical course in medical mycology is given at the C.B.S. The purpose of this course is to give specializing physicians as well as the technical staff members of hospitals an opportunity to become familiar with the fundamentals of mycology and with the methods for isolating, culturing and identifying fungi. Many of the pathogens usually encountered, as well as the common contaminants are studied during this six weeks' course.

Another field of medicine touching mycology is that of fungus allergy. An investigation on the mycological and clinical aspects of bronchial asthma in the Netherlands has recently been carried out by VAN DER WERFF (1958). In a stocktaking of the mould flora in the air of houses and in the open, practically all fungi that have airborne spores may be encountered. Thus the mycological part of the study of fungus allergy has to deal with moulds throughout the whole fungus system.

4. Industrial mycology.

For many centuries moulds have had an evil reputation. Many moulds indeed produce substances in their metabolism that are responsible for the spoilage of foodstuffs and many other materials of organic origin. These moulds keep industries engaged in the search for chemicals controlling mould growth. The discovery of fungi as producers of organic acids, vitamins, antibiotics and growth influencing substances, has directed the interest of industries to new fields of microbiological research and applications. Nowadays a single fungus species may be the centre around which a whole industrial plant has been built up. Consequently the destructive as well as the benificent metabolic products of the fungi are of great economic importance.

All these years a great variety of mouldy items has been sent to the C.B.S. for examination. For example, textiles, varying from a nylon shirt to a fire hose, mouldy paper and ply-wood, a painted panel of a theatre, signalling apparatus accessories, foodstuffs, including imported fruit, even lipsticks were sent in.

The mycologists of the C.B.S. first have to examine whether moulds actually caused the spoilage. In the case of the lipsticks for instance, very minute crystals gave them the appearance of mouldiness. If moulds are indeed the cause, it has to be decided whether it will be necessary to isolate and identify the many different moulds growing on the material. In the case of mouldy books or ammunition boxes, it is of little importance which species of moulds caused the damage, the conditions of storage of the books or the army material are at fault and have to be changed.

If, however, a fruit processing industry has trouble with moulds, exact knowledge of the causal agent is necessary. If only common green Penicillia or Aspergilli are found, something may be wrong with the sterility after processing, such as unsound crown-corks. In case, the ascomycete, *Byssochlamys fulva,* is isolated from the spoilt conserves, the fault must be sought in the sterilizing process itself. The mature ascospores of this fungus can survive a temperature only little below the maximum allowed in processing. As prolonged sterilization at high temperature has to be avoided, because otherwise soft fruits desintegrate by overcooking, great care has to be taken that the lethal temperature for the ascospores of *B. fulva* reaches every part of the contents of the cans. So in this case it is necessary not only to identify the mould which caused the spoilage, but also to know the temperature range within which it can complete its life cycle.

The control of mould growth by means of antiseptic substances has for many years been a subject of much detailed research. The diversity in the tolerance of different fungus species to toxic substances was often overlooked at first. For example salicylic acid which prevents the growth of many species of dark coloured moulds and *Penicillium,* has no effect upon *Aspergillus niger* which can even use this substance as a source of carbon in synthesis. It is advisable that an industry engaged in the production of mould-proofing substances, should first carry out laboratory tests with the different moulds that are liable to attack the product they aim at protecting. In the mycological part of these tests the services of the C.B.S. are often required. The spoilt product, e.g. canvas, cardboard, is sent to the C.B.S. for an analysis of the mould flora. Or, the mould-proofed product, e.g. paint is sent to this laboratory to be exposed to a severe test by moulds known to attack paint, in order to see whether the material is sufficiently protected against these invaders.

The biosynthetic activities of fungi as producers of desirable substances have placed these organisms in a more favourable daylight. Since the discovery of penicillin, moulds have become almost popular. This rapidly developing field of industrial mycology has its applications in many different directions.

Organic acids, such as citric acid (produced by selected strains of *Aspergillus niger*), fumaric acid, d-lactic acid (metabolic products of strains of *Rhizopus oryzae* and *R. nigricans*), itaconic acid (*Aspergillus terreus*) are used in the preparations of foods, of pharmaceuticals, in the production of plastics and many other technical applications. Pharmaceutical industries manufacture vitamins by microbial metabolism: riboflavin or vitamin B_2 is produced by the fungi *Eremothecium ashbyii* and *Ashbya gossypii*, vitamin B_{12} is obtained from *Streptomyces olivaceus* and as a byproduct from the manufacture of antibiotics by *S. griseus* and *S. aureofaciens*. The industrial manufacture of antibiotics started during World War II with the production of penicillin (metabolite of *Penicillium notatum*). Since then, many valuable therapeutics of this class have been discovered, mostly derived from species of the Actinomycetes: streptomycin (produced by *Streptomyces griseus*), chloromycetin (*S. venezuelae*), aureomycin (*S. aureofaciens*), terramycin (*S. rimosus*) to mention only some of the most important. All these substances can only be obtained from organisms that can be grown in artificial culture.

Besides these specific endproducts obtained by microbial metabolism, new biochemical capabilities of fungi have been recently discovered. Fungi and other micro-organisms can effect series of modifications in molecules. The microbial steroid transformations used in the synthesis of cortisone are a very successful example. Much of the research activity on these processes is still hidden behind the walls of industries. The reader is referred to a short survey of this very extensive subject by Professor Kenneth B. Raper (1957) under the suggestive title "Microbes – man's mighty midgets".

Many biochemically important moulds have been found in the groups of Penicillia, Aspergilli, Mucorales, Yeasts and Actinomycetes. The Actinomycetes, although nowadays classified with the bacteria, are also incorporated in the culture collection of the C.B.S. Each new discovery is felt at the C.B.S. in a sudden increase of the orders of a special species or group of species. Last year it was *Gibberella fujikuroi* (an Ascomycete with a *Fusarium* as its imperfect stage), that suddenly came into the limelight as the producer of the growth-influencing substance gibberellin.

How many more species of fungi will in future reveal their still unknown capabilities to the investigating microbiologists! I must confess that I always feel a little sorry, when I read at the end of the description of a new fungus that the material has been deposited in a herbarium. This means the untimely death of the organism of which often only a single specimen was collected and there it is: dry and dead! Why did not the discoverer first pick off a few spores to try to cultivate this fungus? Then the morphological characters could remain preserved in the herbarium and the cultured fungus would allow of an investigation into its biochemical properties.

The C.B.S. contributes to this field of microbiological industry in two ways: on the one hand by collecting, culturing, and then supplying the required species and strains, on the other hand by identifying cultures which, isolated by research investigators, proved to produce substances of economic importance.

Recently a pharmaceutical firm sent to the C.B.S. a fairly detailed description of a *Penicillium*, one of at least 150 species, asking whether our mycologists could name it from this description. A patent for a product of this mould being applied for, they were reluctant to give the organism out of their hands. An exact identification, however, was not possible without seeing the fungus, so it was agreed that the organism should be sent to the C.B.S. provided the material be destroyed immediately after identification, a new culture being promised to the C.B.S. as soon as the patent should have been obtained. This anxiety was unnecessary, because regularly cultures of organisms for which a patent application is in progress, are deposited with the C.B.S. Good care is taken that these cultures are not given out and that their names do not appear in the card index or in the catalogue, until the organism is given free for distribution. The necessity of the exact identification of an industrially important organism is clear from the well-known case of the penicillin producing fungus. THOM (1945) in his paper "Mycology presents penicillin" relates the history of FLEMING's mould. FLEMING tentatively identified his organism as *Penicillium rubrum* and published this name in his first paper (1929) on the discovery of the antibiotic. About a year later Professor HAROLD RAISTRICK was working on the species isolated by FLEMING. Not being a mycologist either, he wisely sent a transfer of this mould to CHARLES THOM, an authority in Penicillia, asking him for an exact identification. THOM then identified it as *P. notatum* and wrote about it (THOM l.c. p. 461–462):

> "The culture received from RAISTRICK was put in the THOM collection as 144.5112.1. The corrected name appears in RAISTRICK's papers and was accepted by FLEMING in his 1932 paper. Nevertheless, requests for *P. rubrum* were frequently received during the several succeeding years. These were usually answered by sending the FLEMING culture and a letter explaining the corrected nomenclature".

The exciting discoveries and the brilliant applications in microbiological physiology sometimes lead to considering the study of taxonomy an oldfashioned subject on its last legs. Owing to increasing specialisation, however, no field of science is independent of any other. The taxonomist guards the other branches of scientific and applied microbiology against much dangerous confusion as to the identity of the organisms they need for their experiments.

Mycology is rapidly developing into an important branch of microbiology. Theoretical and practical training in the morphology and physiology of the fungi will soon have to become part of the curriculum of students in microbiology and applied botany.

THE ACTIVITIES OF THE C.B.S.

Some figures may serve to illustrate to what extent the C.B.S. contributes with the supply of cultures, identifications and advice to these different fields of science and industry.

1. Distribution of cultures.

During 1957, the 50th year of its existence, more than 2.000 cultures, among which 250 yeasts, were distributed. Judging from the pertaining correspondence the orders came from University laboratories or other institutions for scientific research, from phytopathological laboratories and institutions carrying out research for agriculture, horticulture and forestry. Many orders were placed by industries and laboratories engaged in industrial research. Medical institutions sent in requests for cultures. Orders were also received from food inspection services, dairy laboratories, and Indonesians in the Netherlands, who required *Rhizopus oryzae* to ferment soja beans for the production of tempeh (See table 1).

TABLE 1.

Distribution of Cultures in 1957.

	Number of orders	Number of cultures requested
University laboratories	233	924
Phytopathological institutions	110	427
Industries	173	670
Medical institutions	43	124
Miscellaneous	28	88
Total	587	2233

Moreover cultures were sent to the American Type Culture Collection, the Commonwealth Mycological Institute and several other culture collections abroad, to whom the C.B.S. is always glad to send cultures in exchange for their very valuable help in sending to Baarn cultures that are wanted for the C.B.S. collection. Before an order is sent off, each culture has to be checked microscopically and compared to the pertaining literature to ensure the greatest possible guarantee of trueness to species.

Physiological properties of the fungi such as virulence, or the production of special metabolites can never be guaranteed. These properties may change in culture, and to check them each time for all these different products is beyond the possibilities of the culture collection. The work involved would make the costs of such a culture far too high.

2. Growth and maintenance of the culture collection.

In the past year about 200 new species and strains were received from all over the world. Many of them are type cultures of species new to science. All newly received cultures are carefully checked as to their trueness to species, before they are incorporated into the collection. The best culture media and favourable culture conditions of light and temperature have to be tried out. If time permits drawings up to scale are made, to lay down the characteristics of the fungus at the time when it was received.

Concerning general directions for the maintenance of mould cultures the reader is referred to the books of G. Smith (1954), Langeron and Vanbreuseghem (1952) and van der Werff (1958). Only some remarks especially pertaining to the C.B.S. collection will be made here. Most of the stock cultures are transferred 2 to 3 times a year, some groups (*Pythium*, *Phytophthora*, Myxomycetes, many Basidiomycetes) have to be transferred more frequently. Each time two subcultures are made on different media alternatingly so as to give the fungi a periodical change of diet. After having been checked for purity and good growth, the cultures are stored in a room where the temperature is about 18°C. Formerly the stockcultures were transferred by the mycologists, but as the numbers of strains grew and equally so the different tasks of the scientific staff members, technical assistants had to be trained to do the subculturing, carefully supervised by the mycologists. It takes some time to convince a technician that a culture with only a few spores on some scanty hyphae is to be preferred to a whole tube full of luxuriantly growing but sterile mycelium! How to maintain the characteristic sporulation of the moulds in artificial culture is the daily concern of the curator of the collection. Some fungi vary but little in successive transfers, a strain of *Schizophyllum commune* obtained from Kniep in 1920 still develops fruit bodies with lamellae, even on agar slants in tubes. Other fungi again, are unstable and have to be coaxed to produce their fruiting structures by offering them a carefully chosen diet. About 20 culture media are in daily use, 50 other media are regularly prepared. A favourable medium for Basidiomycetes, Penicillia, Aspergilli, and many other Fungi Imperfecti is cherry agar, made from a decoction of the Dutch "May-cherry". Many pycnidial fungi produce fruit bodies readily, when grown on sterilized pieces of lupin stem (pieces of stem about 10 cm long are sterilized in test tubes with 3 ml water), a rather dry medium, poor in nutrients. Prolonged culturing on an unsuitable medium or careless transferring may make a strain atypical and even irreversibly sterile. Some species require light for the development of their fruiting structure e.g. many Ascomycetes, most of the *Helicosporae*, and species of *Pilobus*. To keep the cultures in a vigorous and if possible sporulating state, it is of the greatest importance to choose suitable culture media and environmental conditions.

Often the morphological characters as well as the biochemical properties of a fungus are better preserved in lyophilized condition than in agar cultures. The C.B.S. now aims at keeping all species that can survive the process in lyophilized tubes as well as on agar slants. Moreover one tube of each strain of the collection is stored under mineral oil.

3. Identifications.

In 1957 about 1.000 cultures to be identified were sent in from various parts of the world. These cultures were received from phytopathological laboratories, chemical and pharmaceutical industries, technical institutes, hospitals and laboratories for scientific research, 200 cultures being identified at the yeast division at Delft, 375 strains at the division of medical mycology. Several times species new to science are thus discovered and described by the mycologists of the C.B.S. Interesting strains are incorporated into the collection. The many species present in the culture collection, among which many strains of type value and cultures originally identified by authorities in that particular group, provide a comparative material which is a great help in cases of doubtful and difficult identification.

Not infrequently staff members of the C.B.S. are asked to give expert advice to the court, sometimes to the counsel of insurance companies. If for example a shipload of citrus fruits appears to be spoilt by moulds on arrival, the question arises: who is to pay the damage. Have the conditions during transport favoured mould growth or was the material already mouldy before being packed? For a judge this is an extremely difficult question to answer. Even for experienced mycologists this is often far from easy to decide. It requires not only the knowledge of the exact identity of the attacking moulds but also a thorough insight in their requirements for environmental conditions such as moisture and temperature.

The international relationships of the C.B.S. have been growing steadily during these fifty years. Last year 36 countries contacted this institution for cultures, identifications and advice (See table 2, and fig. 2).

TABLE 2.

Country	Number of requests
Austria	16
Belgium	26
Canada	10
Denmark	18
France	26
Great Britain	79
India	9
Italy	23
Jugoslavia	6
Netherlands	485
Norway	9
Russia	6
South Africa	9
Sweden	15
Switzerland	40
Western Germany	106
USA	105
19 different countries	1 to 5

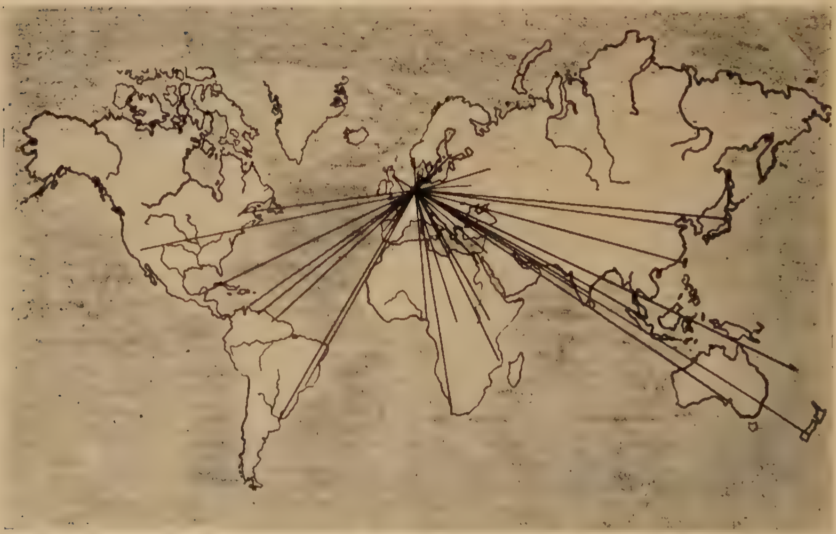

Fig. 2. Outline map illustrating international contacts. The many lines in Europe, the United States and South-Africa overlap, and had to be combined to single lines to the farthest place of contact.

4. Guest workers.

The collection of the C.B.S. offers a unique opportunity to all those who have to practise the identification of moulds. This is also realized in foreign countries. In the past few years two Spanish guest workers stayed at the C.B.S., one working at a pharmaceutical laboratory, the other engaged in industrial research. A German and a Danish phytopathologist came for several weeks, respectively two months, to become acquainted with the taxonomy of moulds. A dermatologist, a veterinary surgeon from Yugoslavia and a Norwegian physician came for a special training in medical mycology.

Unfortunately the C.B.S. has no teaching facilities, and these guest workers as a rule need a great deal of help in order to make their stay profitable. Our mycologists, however, are well aware of the importance of instruction in fungus science, and try to fit in this extra task as far as possible with the current work.

From these data it can be concluded that the C.B.S. has an important task to fulfil in mycological research both in the Netherlands and in many other parts of the world.

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UNTERSUCHUNGEN ÜBER DIE STERILITÄT VON ZWIEBELSAMEN (*ALLIUM CEPA*)

von

W. DAWID[1])

(Eingegangen am 21. August 1958).

PROBLEMSTELLUNG.

In den letzten Jahren erschienen immer wieder Arbeiten, die das Sterilitätsproblem der Pflanzen erneut zur Diskussion stellten (SZILVASI 1942, SCHANDERL 1943, 1947, 1953, TONZIG und BRACCI 1951, 1955, KARDOS und BAGOLY 1955). Diesen und anderen Autoren ist es gelungen, aus verschiedenen Organen verschiedener Pflanzen Bakterien herauszuzüchten. Dabei stellte sich automatisch die Frage nach dem Ursprung dieser Bakterien. Einige Autoren erklären dieses Ergebnis durch eine Primärinfektion der Pflanze von außen (RIPPEL 1940, BURCIK 1948 u.a.). Andere Forscher sind der Auffassung, dass es sich um Spontaninfektionen während des Arbeitens handelt (BORTELS 1941, FISCHER 1948). Eine dritte Gruppe sieht den Ursprung der erhaltenen Bakterien in den Pflanzenzellen selbst und diskutiert die Frage, ob nicht Zellorganelle, wie Mikrosomen, Chondriosomen und Plastiden, umgewandelte Bakterien seien (SCHANDERL 1947, 1950, 1953, KARDOS und BAGOLY 1955, DAWID 1957). Die durch die positiven Versuchsergebnisse ausgelöste Polemik zeigt, wie aktuell und kompliziert das Problem der Sterilität ist (BURCIK 1940, HENNIG und VILLFORTH 1940, SCHAEDE 1940, STAPP 1951, SEYFARTH 1952, KELETI 1954, JUHASZ 1954, NECAS 1956, SOCIAS 1949, GONZALES 1953, SAMISH 1957).

In einer Arbeit über die extrazellulären Entwicklungsmöglichkeiten von Chondriosomen aus Pflanzenzellen berichten KARDOS und BAGOLY (1955), daß es ihnen gelungen sei, in Epidermiszellen der Küchenzwiebel eine aktive Bewegung der Chondriosomen im Cytoplasma zu verfolgen und kinematographisch festzuhalten.

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1) Eynattenerstrasse 59, Aachen.

In Abimpfungen mit Milchsaft der Zwiebel, der die gleichen Chondriosomenformen enthielt wie die Epidermiszellen, konnte ein Bakterienwachstum in 90–95% der Fälle beobachtet werden. Diese Abimpfungen, wiederholt im gleichen Raum durchgeführt, brachten immer die gleiche Bakterienart. Aus Samen vom Stechapfel (*Datura stramonium*) konnte unter sterilen Bedingungen eine Bakterienart isoliert werden, die bis zur 50. Subkultur eine atropinartige Substanz produzierte (Test am Froschherz).

Auf Grund der Tatsache, daß bei den Abimpfungen mit dem Milchsaft der Zwiebel zu verschiedenen Zeiten immer wieder der morphologisch gleiche Bakterientypus erhalten werden konnte, wenden sich Kardos und Bagoly von der Annahme einer ursprünglichen Primärinfektion durch den Boden ab und neigen zur Ansicht, daß es sich um eine zyklische Symbiose von Zelle und Mikroorganismus handle.

Es liegt die Vermutung nahe, daß die coccus- und stäbchenförmigen Chondriosomen in den Samen von *Datura* symbiontische Bakterien sein könnten, die sich kultivieren lassen und eine atropinartige Substanz bilden. In diesem Falle müßte sich das Chondriosom als Symbiont unter gewissen Bedingungen zu definitiven Bakterien zurückverwandeln. Daneben wäre eine zweite Möglichkeit denkbar: Neben den Chondriosomen befinden sich in der Zelle die Symbionten in Bakterienform oder bakterienähnlicher Form.

Falls hier tatsächlich eine Symbiose in den Zellen von *Allium cepa* vorliegt, wobei die Frage der funktionellen biochemischen Beziehungen völlig offenbleibt, wäre zu erwarten, daß der Symbiont bereits in den Zwiebelsamen vorhanden und ebenfalls kultivierbar ist.

Bei den vorliegenden Untersuchungen kam es dem Verfasser darauf an, festzustellen, ob sich aus den Samen der Küchenzwiebel (*Allium cepa*) ein Mikroorganismus züchten läßt, wobei die Frage nach der Beziehung der Chondriosomen zu den eventuell auftretenden Bakterien beantwortet werden sollte.

Material und Methodik.

Bei derartig schwierigen und umstrittenen Untersuchungen ist die Auswahl des Materials und der Methodik entscheidend für die Eindeutigkeit und Wiederholbarkeit der Ergebnisse. Daher seien methodische Einzelheiten genauer dargestellt.

Material.

Als Untersuchungsmaterial dienten Zwiebelsamen (*All.cepa*), Erfurter Dom-Samen Nr. 145, "Zittauer Gelbe". Die Nährlösung wurde nach dem Rezept von Hallmann (1953) hergestellt:

20 ccm Glycerin DAB 6, 10 g Liebig-Fleischextrakt, 10 g Pepton Witte, 5 g NaCl p.a., 1000 ccm Wasser (Glycerin-Bouillon).

Der pH-Wert wurde auf 7,2 eingestellt. Die fertige Glycerinbouillon wurde in Erlenmeyerkolben fraktioniert sterilisiert, nach 14 Tagen von den ausgefallenen Eiweißteilchen abgegossen und in Reagenzgläser zu 6 ccm abgefüllt, anschließend im Dampftopf fraktioniert sterilisiert. Auf dem Boden der Röhrchen war mit der Lupe der sonst feine, störende Niederschlag nicht nachzuweisen.

Als festes Nährsubstrat diente Glycerin-Agar. Die 2%-ige Glycerin-bouillon wurde mit 2% Agar versetzt und in üblicher Weise weiterverarbeitet.

Die Samen wurden in einer Bäderreihe desinfiziert, die aus folgenden Lösungen bestand:

Bad 1 10%-ige Chlorkalklösung
Bad 2 5 %-ige wässrige Chloraminlösung
Bad 3 konz. alkohol. Chloraminlösung.

Zur Vitalfärbung von Zellmaterial und flockigem Niederschlag diente eine Janusgrün B – Lösung (Hollborn), 1 : 10 000 verdünnt in Leitungswasser. Ausstrichpräparate wurden mit Gentianaviolett oder Safranin (Hollborn) gefärbt.

Methodik.

Die mikroskopische Untersuchung der Zwiebelsamen wurde in üblicher Weise vorgenommen. Handschnitte und Quetschpräparate wurden 20–30 Min. mit Janusgrün B vitalgefärbt, danach mit dem Deckglas abgedeckt und beobachtet.

Zur Beobachtung unter sterilen Bedingungen im "Hängenden Tropfen" (HT) wurden hohlgeschliffene Objektträger in Petrischalen sterilisiert, mit einem Ring steriler Vaseline (Tube) versehen, das Untersuchungsmaterial auf sterile Deckgläschen gebracht und HT hergestellt. Als Untersuchungsflüssigkeit wurde Glycerin-bouillon benutzt. Zur Aufbewahrung dienten sterile feuchte Kammern.

Die Zwiebelsamen wurden wie folgt desinfiziert: Die Desinfektionslösungen wurden 24 Stunden vorher bereitet, die Chlorkalklösung kurz vor Gebrauch mit HCl frisch aktiviert. Nachdem etwa 20–30 Samen in ein Mullsäckchen gebunden waren, wurden sie zuerst kurz in Äther, danach kurz in 40%-igen Alkohol getaucht, damit die Desinfektionslösungen die feingerillte Samenschale benetzen konnten. Das Säckchen wurde mittels eines Fadens für je 5 Min. unter Schütteln in die Kolben der Bäderreihe gebracht. Aus Bad 3 kam der Beutel in eine bereitstehende Petrischale (steril), wo unter leichtem Anheben des Deckels mit einer sterilen Pinzette und sterilen Schere der Beutel geöffnet wurde. Die noch benetzten Samen wurden mit einer jeweils frischgeglühten Pinzette über der Flamme in die Bouillonröhrchen ganz oder zerquetscht übertragen. Die beimpften Röhrchen wurden bei 18°C. 8 Wochen lang bebrütet.

VERSUCHSREIHEN.

I. Mikroskopische Analyse des Zwiebelsamens.

Die Oberfläche der Samenschale ist leicht gefaltet und fein gerillt. Handschnitte von trocknen und gequollenen Samen zeigten folgendes Bild: Die sehr starke Samenschale ist am spitzen Ende leicht gerissen und öffnet sich beim Durchtritt des Keimlings. Nur diese Stelle käme für eine innere Infektion des Samens in Betracht. Im Endosperm eingebettet liegt fast spiralig gewunden der Embryo (Bild 1).

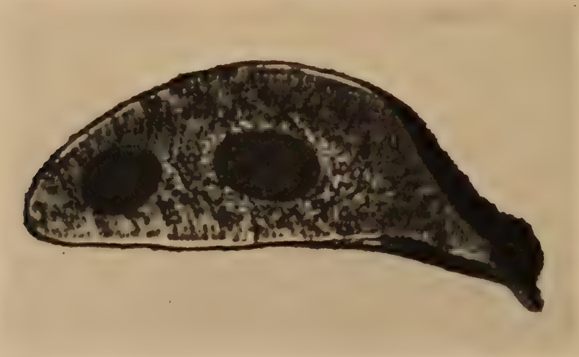


Bild 1. Zwiebelsamen quer, 25×.

Die Endospermzellen sind prall mit eiweiß- und fettartigen Reservestoffen angefüllt. Die sehr stark verdickten Zellwände zeigen einfache Tüpfel. In Dünnschnitten des Endosperms wurde beobachtet, daß die Eiweißstoffe infolge der Quellung aus den Zellen traten und feine Gerinsel ("Filigranstrukturen") bildeten. Mit Janusgrün B zeigten sowohl vereinzelte Chondriosomen als auch diese Eiweißkomplexe die gleiche Färbbarkeit. Auf diese Tatsache, daß auch eine Adsorption des Farbstoffes an Plasmaeiweißkörper erfolgt und daher nicht alle mit Janusgrün B angefärbten Partikel Chondriosomen sind, hat u.a. SHOWACRE (1953) aufmerksam gemacht.

Ein völlig anderes Bild zeigten die Embryozellen: Sie sind von einer dünnen Membran umgeben, das Innere ist fast nur von Chondriosomen angefüllt (Bild 2). Die Beobachtungen zeigen, daß die Chondriosomen in drei Formen vorhanden sind: Coccus, Diplococcus und Stäbchen. Die Hauptmasse bilden die coccusförmigen, sphärischen 1,2μ großen Chondriosomen. Sie sind mit den von PERNER und PFEFFERKORN (1953) beschriebenen Chondriosomen der Zwiebelepidermis identisch.

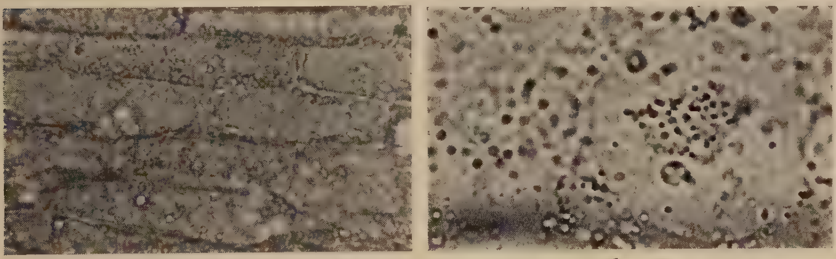

a b

Bild 2. Epidermiszellen des Zwiebelembryos, lebend.
a. ungefärbt, 600 ×.
b. gefärbt mit Janusgrün B, 1350 ×.

Sehr häufig sind die Diplococcusformen, die in diesen Embryozellen zahlenmäßig stärker zu finden waren als in den Epidermiszellen. Stäbchenförmige Chondriosomen von 4μ Länge waren schwer zu sehen.

Von herauspräparierten Embryonen wurden Quetschpräparate im HT hergestellt. Bei der Beobachtung zahlreicher Präparate zeigte sich, daß große Mengen coccusförmiger Chondriosomen infolge der Brown'schen Molekularbewegung sich zitternd bewegten (Bild 3) und daß unter ihnen stäbchenförmige deutlich sichtbar wurden (Bild 4). Diese, an einem oder beiden Enden leicht gekrümmten Chondriosomen bewegten sich, unabhängig von der

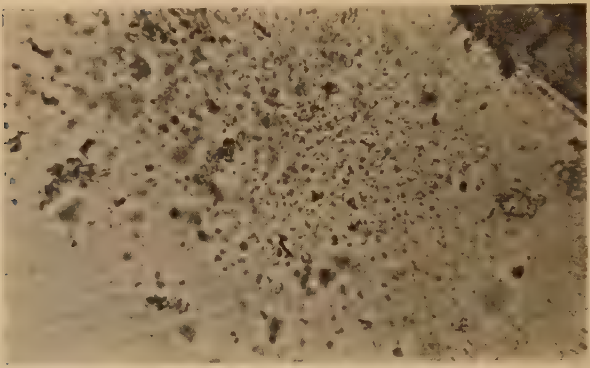


Bild 3. Chondriosomen aus dem Zwiebelembryo, lebend, ungefärbt, 600 ×.

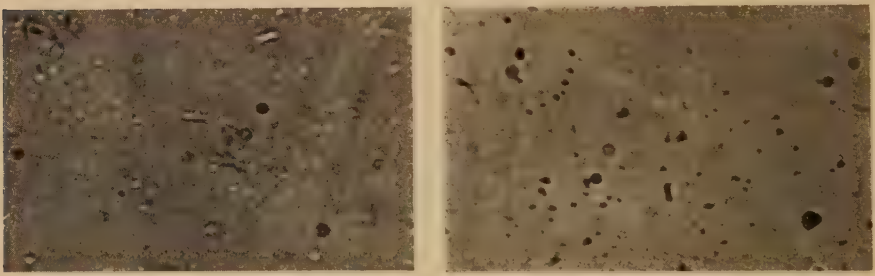


Bild 4. Stäbchenförmige Chondriosomen, lebend, 900 ×.
Bild 5. Chondriosomen, Janusgrün B, 600 ×.

Molekularbewegung, ruckweise vorwärts, wobei der Körper gekrümmt und nicht selten um die Längsachse gedreht wurde. Besonders gut konnte diese aktive Bewegung stäbchenförmiger Chondriosomen gesehen werden, wenn ein Eiweißpartikel als Hindernis zu umgehen war.

Nach oben angegebener Methode wurde Zwiebelsamen desinfiziert und in Kolben mit sterilem Wasser gründlich ausgewaschen. Mit frischgeglühter Pinzette und Lanzettnadel wurden die Embryonen in sterilen Petrischalen herauspräpariert und 1 mm große Stücke unter sterilen Bedingungen zu HT verarbeitet. Als Untersuchungsflüssigkeit diente Janusgrün B oder Glycerinbouillon. Die HT wurden 14 Tage hindurch beobachtet. In Präparaten mit Janusgrün B kam es zu einer guten Vitalfärbung (Bild 5), und die Eigenbeweglichkeit der stäbchenförmigen Chondriosomen konnte bestätigt werden. Nach einigen Tagen waren diese Präparate infolge eingetretener Degradationserscheinungen für Dauerbeobachtungen unbrauchbar. Als geeignetere Untersuchungsflüssigkeit erwies sich die Glycerinbouillon. Die Chondriosomen konnten 14 Tage hindurch unverändert gut beobachtet werden. In diesen HT konnte wiederholt festgestellt werden, daß sich Diplococcusformen "teilten" d.h. in zwei coccusförmige Chondriosomen zerfielen. Stäbchenförmige Chondriosomen zerfielen in zwei, selten drei Teilstücke, die noch eine Zeitlang zusammenhingen und sich dann trennten. Es war nicht möglich, neben dieser Fragmentation eine regelmäßige Teilung oder Vermehrung der Chondriosomen festzustellen. Die Übereinstimmung der Chondriosomen mit Stäbchenbakterien bleibt auf morphologische Ähnlichkeit beschränkt.

Durch die Desinfektion war eine Schädigung der Zellen und der Chondriosomen nicht eingetreten. In keinem der HT kam es zu irgendeiner Bakterienentwicklung.

II. Vorversuche.

Keimschädigung durch Desinfektion.

Um Aufschluß zu erhalten, ob durch die Desinfektion in der Bäderreihe Keimschädigung der Zwiebelsamen eintritt, wurden Keimproben vorgenommen. In mehrere Petrischalen mit geglühtem und getränktem Seesand wurden je 100 Samen, unbehandelt oder desinfiziert, gelegt. Nach 20 Tagen wurde ausgezählt:

Unbehandelte Samen	Desinfizierte Samen
70% gekeimt	53% gekeimt
30% verschimmelt	47% nicht gekeimt.

Daraus ergibt sich eine Keimschädigung durch Desinfektionsmittel von 17%.

Test auf Wirksamkeit der Bäderreihe.

Das mikroskopische Bild der Samenschale (Bild 1) ließ vermuten, daß sich in den feinen Rillen der Oberfläche Bakterien oder Pilzsporen befinden und während der Desinfektion unbeschädigt bleiben. Daher wurde die Bäderreihe vor Anwendung zur Desinfektion von Samen auf ihre Wirksamkeit geprüft: Je 1 desinfizierter Same wurde in Bouillonröhrchen gebracht und 14 Tage bei 18°C. bebrütet. Es stellte sich kein Bakterienwachstum ein.

Zur Kontrolle über das eventuelle Eindringen von Keimen in die Samenschale während der Quellung wurden unbehandelte Samen in eine Bouillonkultur von Bakterien, *Bac. mesentericus* bzw. *Micrococcus luteus* gebracht und nach 20 Min. an der Luft getrocknet. Parallel dazu wurden desinfizierte und ausgewaschene Samen getrocknet und in den gleichen Bakterienkulturen getränkt. Nach dem Trocknen wurden die Samen durch die Bäderreihe gebracht und in Bouillonröhrchen abgeimpft. Nach 14 Tagen bei 18°C. zeigte kein Röhrchen irgendein Wachstum von Mikroorganismen.

Damit war der Beweis erbracht, daß die benutzte Bäderreihe mit den Desinfektionszeiten zur äußeren Desinfektion von Zwiebelsamen geeignet ist.

Untersuchung der Mikroflora der Samenschale.

Die an der Samenschale anhaftenden Mikroorganismen wurden folgendermaßen untersucht: Unbehandelte Zwiebelsamen wurden einzeln in 20 Bouillonröhrchen gebracht. Nach 5 Tagen waren alle Kulturen stark getrübt, teils molkig-weiß. 16 Röhrchen fluoreszierten an der Oberfläche gelblichgrün, 4 zeigten eine stark weißliche Färbung. In allen Kulturen war ein mehr oder weniger stark entwickeltes Pilzmycel vorhanden.

Demnach sind etwa 80% der Samen mit Fluoreszenten behaftet. Von diesen Kulturen wurden Platten gegossen, und die fluoreszierenden Kolonien wurden abgeimpft. Die Bakterien sind Kurzstäbchen (2,5μ), meist paarig. Die 15 isolierten Stämme sind morphologisch identisch und scheinen zu *Ps. fluorescens* zu gehören (Bild 6). Weißlichgraue, glänzende, unregelmäßige Kolonien traten zahlenmäßig an zweiter Stelle auf der Platte auf. Es waren Lang-

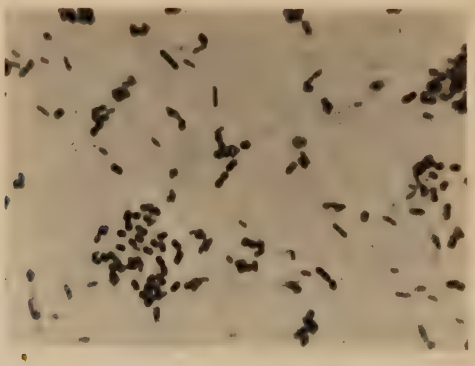


Bild 6. Fluoreszenten der Samenschale, Methylenblau, 900 ×.

Bild 7. Stäbchenbakterien der Samenschale, Methylenblau, 900 ×.

stäbchen von 4,5μ Länge, die zur Sporenbildung befähigt waren (Bild 7). Die Bouillon wurde stark getrübt, und ein grauweißes Häutchen bildete sich.

Beide Arten scheinen typische Vertreter der Mikroflora der Samenschale zu sein. Zum eventuellen späteren Vergleich wurden sie weitergezüchtet.

Den Schimmelpilzarten wurde keine Beachtung geschenkt, da sie in diesem Zusammenhang nicht interessierten.

III. Abimpfung desinfizierter Zwiebelsamen.

Nach diesen Vorversuchen wurden nun Abimpfungen äußerlich desinfizierter Zwiebelsamen in Glycerinbouillonröhrchen vorgenommen. Dabei wurde in zweifacher Weise verfahren: Desinfizierte, ganze Samen wurden abgeimpft, um sterile Keimlinge zu erhalten. Zum anderen wurden Samen nach der Desinfektion mit einer frischgeglühten Pinzette beim Übertragen in der Flamme zerquetscht. Dadurch sollte die Möglichkeit zum leichteren Austritt von Chondriosomen und eventueller Keime aus dem Gewebe gegeben werden.

Abimpfung ganzer Samen.

Je ein desinfizierter Same wurde in 100 Glycerinbouillonröhrchen gebracht. Bei 18°C. wurden die Kulturen bebrütet. Nach 4 Tagen waren 3 Röhrchen am Flüssigkeitsrand verschimmelt (*Penicillium* spec.). Diese Röhrchen wurden verworfen. Nach 10 Tagen keimten die ersten Samen aus. Nach 20 Tagen trat erstmals vereinzelt eine leichte Trübung in den Röhrchen ein, die von den auf den Boden liegenden Samen ausging. Nach 8 Wochen konnte folgendes Ergebnis festgestellt werden:

Von 100 Abimpfungen 28 bakterienpositiv = 28%
3 verschimmelt = 3%
69 ungetrübt = 69%.

Die bakterienpositiven Röhrchen zeigten eine mäßige bis starke weißliche Trübung. Auf der Oberfläche wurde ein dünnes, weißliches Häutchen gebildet mit einer stearinartigen Halskrause. Auf dem Boden war ein schmutziggraues Sediment. Unter dem Mikroskop zeigten sich einheitliche Langstäbchen (3,5μ), die sich gram-negativ färbten. Auf Glycerinagar-Platten wuchsen kleine, unregelmäßige, grauweiße, glänzenden Kolonien. Sie waren alle einheitlich und zeigten mikroskopisch, daß die Bakterien identisch waren (Bild 8). In den positiven Kulturen waren nur zwei Samen ausgekeimt. Die Keimlinge waren bald zersetzt.

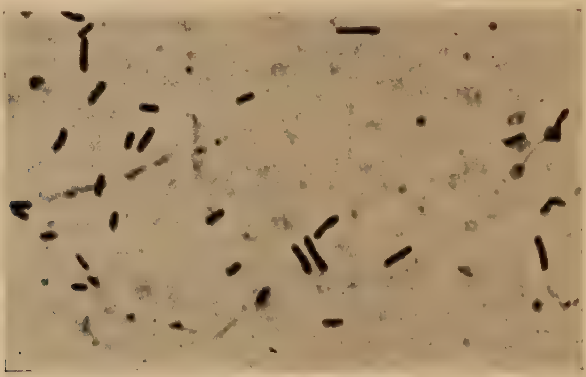
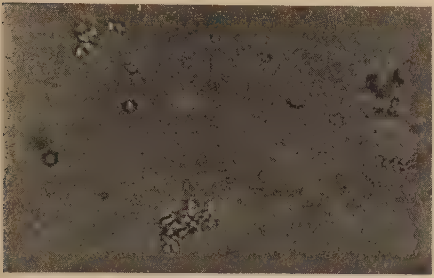


Bild 8. Stäbchenbakterien, isoliert aus Zwiebelsamen, Methylenblau, 1350×.

In den 69 klargebliebenen Röhrchen waren 18 Keimlinge ausgewachsen mit einer durchschnittlichen Länge von 4 cm. In 13 Röhrchen, teils mit, teils ohne Keimling, war ein feines Gerinsel am Boden zu sehen. Es zeigte sich, daß dieser Bodensatz aus kleinsten feinstrukturierten Eiweißkomplexen mit vereinzelten Chondriosomen bestand (Bild 9). Mit Janusgrün B färbten sich beide Bestandteile.

Alle klargebliebenen Röhrchen wurden zur Abtötung der Keimlinge bzw. Samen kurz über der Flamme erhitzt, um ein eventuelles Symbioseverhältnis zwischen Zelle und Bakterien zu lösen (Schanderl 1947). Wegen einer Infektionsgefahr wurden die Keimlinge nicht mit einem geglühten Skalpell zerschnitten (s. Fischer 1948). Nach 14 Tagen zeigten die Röhrchen dasselbe unveränderte Bild.



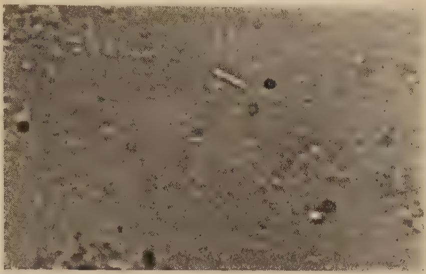


Bild 9. Eiweissstrukturen aus dem Bodensatz zerquetschter Zwiebelsamen, 900 ×.

Bild 11. Stäbchenförmiges Chondriosom aus dem Bodensatz zerquetschter Zwiebelsamen, ungefärbt, 1350 ×.

Darauf wurden sie nochmals kurz erhitzt und bebrütet. Eine Trübung als Anzeichen eines Wachstums von Bakterien blieb aus. Lediglich die feinen Gerinselflocken auf dem Grund der Reagenzgläser waren zahlreicher und zeigten mikroskopisch dasselbe Aussehen.

Während der 12 wöchigen Beobachtungszeit waren nur 28% aller Röhrchen bakterienpositiv.

Abimpfung zerquetschter Samen.

Nach der oben beschriebenen Methode wurden 100 Glycerinbouillonröhrchen geimpft und bei 18°C. bebrütet. In mehreren Röhrchen lagen zwei Samenstücke auf dem Boden. In 5 Röhrchen wuchsen nach 3 Tagen an der Oberfläche Schimmelpilze aus. Nach 13 Tagen keimten einige Samen. Nach 10 Tagen zeigten einige Röhrchen eine leichte Trübung. Auffallend war ein feiner, flockiger Bodensatz in allen Röhrchen. Nach 8 Wochen zeigte sich folgendes Ergebnis:

Von 100 Abimpfungen 36 bakterienpositiv = 36%
5 verschimmelt = 5%
59 ungetrübt = 59%

Bei der Untersuchung der Bakterienkulturen zeigte sich eine fast gleichstarke Trübung, verglichen mit Abimpfungen ganzer Samen. An der Glaswand erschienen die gleichen, stearinartigen Krausen mit den dünnen Häutchen. Das Sediment war schmutziggrau. In den Ausstrichpräparaten konnten ebenfalls morphologisch gleiche Langstäbchen festgestellt werden. Sie waren gram-negative Nichtsporenbildner (vergl. Bild 8). Auf Glycerinagarplatten wuchsen sie in glänzenden, grauweißen, unregelmäßigen Kolonien gleichen Typus.

In allen 59 ungetrübten Röhrchen fand sich um die Samen ein feiner, flockiger Niederschlag, der bei leichtem Schütteln schleierartige Wolken aufwirbeln ließ. Der Verdacht auf ein langsam wachsendes Bakterium lag nahe. Bei der mikroskopischen Beobachtung zeigte sich, daß diese Flocken nichts anderes als riesige Mengen feiner Eiweißkomplexe ("Filigranstrukturen") und Chondriosomen sind. Mit Janusgrün B färbten sich beide Bestandteile intensiv (Bild 10). Unter sterilen Bedingungen wurden HT aus derartigem Bodensatz hergestellt und mehrere Tage hindurch beobachtet. Die coccusförmigen Chondriosomen, teils Diplostadien, veränderten sich nicht. An mehreren stäbchenförmigen Chondriosomen konnte beobachtet werden, wie sich diese "querteilten". Die zwei oder auch drei entstandenen Fragmente bleiben, ähnlich wie bei Stäbchenbakterien, zusammenhängen (Bild 11). Eine regelmäßige, bakterienähnliche Vermehrung war nicht zu beobachten. Die Eiweißgerinsel, die manchmal ähnliche Formen zusammenhängender Coccen und Stäbchen darstellten, blieben während der Beobachtung unverändert. Diese Strukturen erwiesen sich als nichts anderes als gequollenes Reserve- und Plasma-eiweiß der zerstörten Endospermzellen. Eine Entwicklung coccus- oder stäbchenförmiger Chondriosomen zu Bakterien war im HT nicht nachzuweisen.

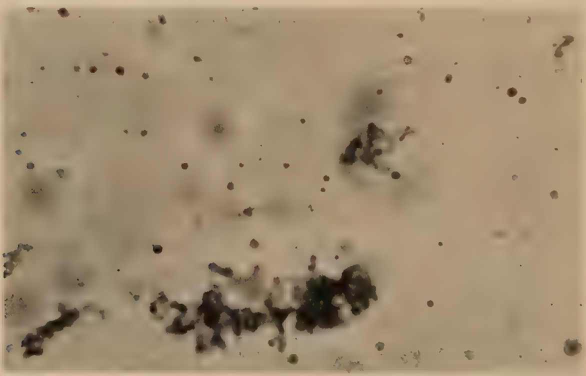


Bild 10. Eiweissstrukturen und coccusförmige Chondriosomen aus dem Bodensatz zerquetschter Zwiebelsamen, Janusgrün B, 900 ×.

In 12 klaren Röhrchen waren die Samen ausgekeimt bis zu 3 cm Länge. Durch Hitzetod und Bebrütung kam es nicht zum Auswachsen von Bakterien.

BESPRECHUNG DER ERGEBNISSE.

Die Tatsache, daß aus desinfizierten Zwiebelsamen in 28–36% der Fälle ein einheitlicher Mikroorganismus gezüchtet werden konnte, stellt nun die Frage, woher der Mikroorganismus stammt.

Die ersten Bakterien kamen in den Abimpfungen nach 10 bzw. 20 Tagen zur Entwicklung. Daraus kann geschlossen werden, daß sie aus dem Inneren des Samens stammen und erst nach Quellung und Zerstörung des inneren Zellgefüges frei wurden. Die Abimpfungen mit zerquetschten Samen bestätigen diese Annahme, denn gegenüber den Abimpfungen mit ganzen Samen begann hierbei das Bakterienwachstum etwa 10 Tage früher und häufiger.

Zunächst soll die Frage erörtert werden, ob sich eine Beziehung zwischen den Chondriosomen des Zwiebelsamens und den aus ihnen erhaltenen Bakterien nachweisen läßt.

Den Stäbchenbakterien sind mikroskopisch analog die stäbchenförmigen Chondriosomen, die in den embryonalen Zellen, in Quetschpräparaten von Keimlingen und im flockigen Bodensatz der meisten Abimpfungen zu beobachten waren. Diese stäbchenförmigen Chondriosomen zeigten eine aktive Bewegung, wie sie von FREDERIC und CHEVREMONT (1952), KARDOS und BAGOLY (1955) u.a. beobachtet und kinematographisch dargestellt worden ist. Im HT konnte zwar eine Teilung der Chondriosomen in Form einer Fragmentation einige Male gesehen werden, aber eine regelmäßige Durchschnürung und Vermehrung, wie sie den Bakterien eigen ist, war nicht festzustellen. Bei einigen diplococcusartigen Chondriosomen war die "Teilung" nur eine Trennung beider sphärischen Chondriosomenteile. Der Begin einer neuen "Teilung" und der damit verbundenen Durchschnürung wurde nicht beobachtet. Über derartige Fragmentationen bei Chondriosomen liegt eine Reihe von Arbeiten vor (WEATHERFORD 1953, DANNEEL und GÜTTES 1951, FREDERIC und CHEVREMONT 1952, u.a.).

So konnte in zahlreichen HT unter sterilen Bedingungen eine Entwicklung irgendwelcher Stäbchenbakterien aus coccus- oder stäbchenförmigen Chondriosomen nicht beobachtet werden. Auch kein anderes Bakterienwachstum wurde gefunden. Daß ein Übergang von coccus- zu stäbchenförmigen Chondriosomen als Folge des Wachstums möglich ist, wird von GUILLIERMOND (1941) beschrieben.

Diesen eigenen Beobachtungen stehen entgegengesetzte Untersuchungsergebnisse gegenüber, nach denen es gelungen sein soll, Chondriosomen zu züchten (Hurst und Strong 1932, Seyfarth 1952) oder eine Entwicklung von Chondriosomen zu Bakterien zu beobachten (Schanderl 1950).

Gegen eine Bakteriennatur der Chondriosomen spricht auch die lamellenartige Feinstruktur der stäbchenförmigen Chondriosomen, wie sie von Palade (1953), Sjöstrand (1953), von Wettstein (1954) u.a. elektronenoptisch gesehen und wiedergegeben wurden. Eine derartige Innenstruktur ist von Bakterien bisher nicht bekannt.

Die beobachteten feinstrukturierten Eiweißkomplexe, die oft coccus- und stäbchenförmige Strukturen zeigten ("Filigranstrukturen"), erwiesen sich als Eiweißpartikel, die als Ergebnis der Quellung von Reserveeiweiß der Endospermzellen zu betrachten sind. In den Abimpfungen mit zerquetschten Samen traten sie daher in jedem Röhrchen als flockiger Niederschlag auf. Sie stehen weder in Beziehung zu den Chondriosomen noch zu den erhaltenen Bakterien. Auch eine Beziehung zwischen den Bakterien der Samenschale und den aus den Samen isolierten Bakterienart war nicht zu finden.

Die eigenen Ergebnisse stehen im Widerspruch zur Auffassung Schanderl's (1947) von der Symbiose zwischen Zellplasma und Chondriosomen (= domestizierte Bakterien als Symbionten). Durch das Zerquetschen der Samen wurden Embryozellen zerstört und Chondriosomen freigesetzt. Sie hätten im abgestorbenen Zellplasma und in der Glycerinbouillon Nährstoffe für eine Weiterentwicklung zur Verfügung gehabt. In den insgesamt 30 Kulturen mit sterilen Keimlingen sind vor dem Erhitzen 12 durch kräftiges Schütteln abgebrochen. Diese offenen Bruchstellen haben auch die Möglichkeit zum Austritt von Chondriosomen gegeben. Auch nach Abtötung durch zweimaliges Erhitzen wurde kein Bakterienwachstum ausgelöst.

Bei der Annahme, daß die Bakterien durch eine Infektion aus dem Boden in die Mutterpflanze eingedrungen seien, bleibt die Frage offen, warum aus der Vielzahl der in der Rhizosphäre der Zwiebel vorkommenden Bakterienarten nur eine Art in der Lage ist, die gesamte Pflanze zu infizieren.

Die Annahme, daß es sich hierbei um eine Art Symbiose zwischen Zwiebelzellen und Bakterien handle, kann zunächst nur durch den identischen Mikroorganismus gestützt werden, wie er in den eigenen Versuchen aus den Zwiebelsamen und von Kardos und Bagoly aus dem Milchsaft der Zwiebel erhalten wurde. Aus dem Vergleich der Angaben scheinen die beiden Bakterienarten morphologisch identisch zu sein (Bild 12). Wenn hier eine echte Symbiose vorliegt, so ist zu fragen, warum der Symbiont in den Zellen mikroskopisch nicht nachweisbar und nur in 28 bis 36% der Fälle kultivierbar ist. Anhaltspunkte über eine biochemische und physiologische Beziehung zwischen Zwiebelzelle und Bakterium liegen nicht vor.

Es bleibt die Möglichkeit zu prüfen, ob der erhaltene Mikroorganismus als Parasit in der Blüte der Zwiebelpflanze vorkommt und beim Befruchtungsprozeß in die Samenanlage einwandert.

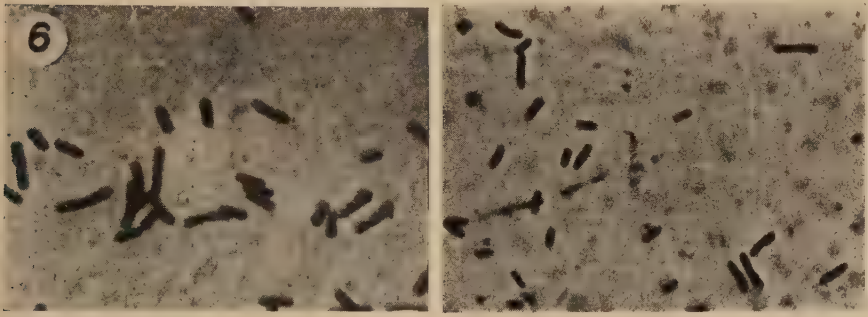


Bild 12. Stäbchenbakterien isoliert aus

a. Milchsaft der Zwiebel nach KARDOS und BAGOLY (1955) gefärbt mit Janusgrün B, 1000 ×.

b. Zwiebelsamen, Gentianaviolett, 1350 ×.

Zusammenfassung.

Die vorliegenden Untersuchungen wurden in Anlehnung an die Untersuchungen von KARDOS und BAGOLY (1955) über die extrazellulären Entwicklungsmöglichkeiten von Chondriosomen der Küchenzwiebel (*Allium cepa*) durchgeführt. Dabei wurde die Frage gestellt, ob äußerlich desinfizierte Zwiebelsamen Bakterien enthalten. Es wurden folgende Ergebnisse erzielt:

1. In Quetschpräparaten aus Embryostücken wurden große Mengen coccus- und stäbchenförmige Chondriosomen gefunden. Die stäbchenförmigen Chondriosomen zeigten neben der Brown'schen Molekularbewegung eine selbständige, aktive Bewegung.
2. Im Hängenden Tropfen wurde unter sterilen Bedingungen in einigen Fällen eine Fragmentation stäbchenförmiger Chondriosomen, eine Trennung der Diplococcusformen in zwei sphärische

Chondriosomen beobachtet. Eine Teilung oder Vermehrung der Chondriosomen in der Art, wie sie Bakterien eigen ist, wurde nicht festgestellt.

3. In Glycerinbouillon abgeimpfte, äußerlich desinfizierte, ganze Zwiebelsamen brachten in 28%, desinfizierte zerquetschte Zwiebelsamen in 36% der Röhrchen Bakterien. Die erhaltenen Bakterien waren morphologisch untereinander gleich und gehören zu einer Art. Sie sind 3,5μ lang, gram-negativ und bilden keine Sporen.
4. Die aus den Zwiebelsamen erhaltenen Bakterien scheinen mit den von KARDOS und BAGOLY aus dem Milchsaft der Zwiebel gezüchteten Bakterien identisch zu sein.
5. Der Nachweis einer Entwicklung von Chondriosomen zu Bakterien konnte nicht erbracht werden.

Summary.

In the present experiments the question whether desinfected onion seeds (*Allium cepa*) are sterile or not has been examined.

It was established that the embryonic cells contain a large quantity of mitochondria – both coccoid and rod shaped. A very active movement of the rod shaped mitochondria was observed in "hanging drops" kept in sterile conditions – like it was established by KARDOS and BAGOLY (1955). In some cases a fragmentation of diplococcoid and rod shaped mitochondria was noted, but no division and no multiplication usual for bacteria.

Desinfected onion seeds kept inoculated in glycerine bouillon produced 28% bacteria in whole seeds and 36% bacteria in crushed seeds. The resulting bacteria were morphologically alike: length 3.5μ, gram-negative, no spore formation. The rod shaped bacteria obtained from onion seeds appear to be identical with those obtained by KARDOS and BAGOLY from onion juice.

A change from mitochondria into bacteria has not been observed. By the above method it was not possible to decide whether the resulting bacteria are present in the onion seeds in symbiosis or as parasites.

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THE REITER PROTEIN COMPLEMENT FIXATION TEST IN EXPERIMENTAL SYPHILIS

by

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In previous papers we studied the significance of a complement fixation test using as antigen the protein fraction of the Reiter treponeme (RPCF test) in the serodiagnosis of the treponematoses (DE BRUIJN 1957, DE BRUIJN and BEKKER, 1957, BEKKER 1958). From these studies it appeared that the RPCF test is a dependable test in the serology of the treponemal infections as the specificity of this test is only slightly less than that of the treponemal immobilization (TPI) test and the sensitivity at least equals that of the TPI test, provided that an adequate technique of complement fixation is used (BEKKER 1959).

The close parallelism between the RPCF and the TPI test and the observation that in treated cases of syphilis the RPCF test seemed to be more sensitive than the other serological tests led us to the study of the antibody pattern of the RPCF test in comparison with those of the TPI test and Kolmer's cardiolipin complement fixation test, especially after treatment.

For this purpose experimentally infected rabbits were used as the data on syphilitic patients suggest that the pattern in man is similar in its essential features to that in the experimental infection.

MATERIAL AND METHODS.

Rabbits were inoculated intratesticularly with a suspension containing 5–10 × 10^6 *T. pallida*, Nichols strain. Before inoculation all rabbits showed negative TPI, RPCF and Kolmer tests. At varying intervals after the development of the orchitis penicillin treatment was given. Each rabbit received 60.000 units of procaine penicillin intramuscularly for 7 consecutive days. TPI, RPCF and Kolmer tests were performed before and at regular intervals after treatment.

The TPI test technique as described by NELSON and DIESENDRUCK has been followed with minor modifications (BEKKER and ONVLEE 1955). The RPCF tests were performed either with the technique of DE BRUIJN (1958) or with Kolmer's technique with $1\frac{1}{2}$ exact unit of complement as described by PORTNOY and MAGNUSON (1956). The rabbit sera were inactivated for 30 minutes at 63° C.

RESULTS.

In a series of 5 rabbits the appearance of the cardiolipin and treponemal antibodies after the experimental infection with virulent treponemes was studied. Approximately 7–8 days after the inoculation the Kolmer test became positive, shortly afterwards followed by the RPCF and TPI tests. Within 14 days after infection all tests were positive. This is in accordance with the observations in cases of human primary syphilis in which the TPI test may be negative and the usual tests already positive.

In an other series of 5 rabbits treatment was started within 3 weeks after the development of the orchitis. In all cases the Kolmer tests became negative in 4–6 weeks, the TPI tests in 6–10 weeks and the RPCF tests in 10–20 weeks.

In a following series of 5 rabbits treatment was given not before 3 months after the development of the orchitis. In 2 of the rabbits all tests remained positive after 40 weeks showing no noticeable decline in titre. In the other 3 rabbits only the Kolmer tests became negative after 4 weeks, the TPI and RPCF tests remaining still positive after 45 weeks.

DISCUSSION.

The results given above confirm the data on treated syphilitic patients obtained in our previous studies in that on the average the sensitivity of the RPCF test is on the same level as that of the TPI test, sometimes the RPCF test being even more sensitive.

As is the case with the TPI test the duration of a positive RPCF test depends on the stage of disease at which treatment was given.

The longer the duration of infection before treatment the longer the RPCF test remains positive. Similar to the TPI test the RPCF test is almost invariably positive in the treated late stages of treponemal infection in spite of therapy. Consequently the greatest value of the RPCF test lies in its use as an aid to the routine serological diagnosis rather than as a test of cure.

Summary.

The pattern of the antibody reacting in the Reiter protein complement fixation (RPCF) test was studied in experimental syphilis of the rabbit in comparison with the patterns of reagin and immobilisin. In accordance with the data on syphilitic patients it was found that the RP antibody appears in the rabbit sera after reagin but before or simultaneous with immobilisin.

After treatment reagin is the first to disappear, followed by the immobilizing antibody and later by the RP antibody. Like immobilisin the duration of the occurrence of the RP antibody in the serum depends on the stage of the disease at which treatment was given. The more time elapses between infection and treatment the longer the RPCF test remains positive.

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(National Institute of Public Health, Utrecht, Netherlands).

INVESTIGATIONS INTO THE ANTIGENIC STRUCTURE OF THE REITER STRAIN OF *TREPONEMA PALLIDUM*

II. THE COMPLEX NATURE OF THE PROTEIN FRACTION[1])

by

J. H. DE BRUIJN

(Received December 18, 1958).

INTRODUCTION.

In the first paper of this series the presence of the 'ubiquitous lipid' in the protein fraction of the Reiter strain of *Treponema pallidum* has been demonstrated (DE BRUIJN, 1958b). This finding may account for the high phosphor content of protein antigen, recorded by D'ALESSANDRO and DARDANONI (1953). The analysis figures obtained by these authors justify the assumption that the protein antigen is a lipopolysaccharide-protein complex. As there is no antibody to the polysaccharide fraction of the Reiter treponema in syphilitic serum (D'ALESSANDRO, ODDO and DARDANONI, 1950), an anti-protein-antigen rabbit serum is used to study the presence of a polysaccharide component in protein antigen. Antigen, heated at 78° C. for 1 hour, still reacts with this antiserum, although to a lesser degree than before heating, whereas its reactivity with human syphilitic serum is completely destroyed. Although this points to the presence of a heat-stable, presumably polysaccharide, component in protein antigen, this was thought impossible by D'ALESSANDRO and DARDANONI (1953) because of the susceptibility of the antigen to N sodium hydroxide and its precipitation by trichloroacetic acid, both reagents being applied to the preparation of the polysaccharide fraction from the whole Reiter organisms (D'ALESSANDRO, ODDO, COMES and DARDANONI, 1949).

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[1]) Part I: Antonie van Leeuwenhoek **24**, 253, 1958.

In the present study the method of RAISTRICK and TOPLEY (1934), already applied to the Reiter treponema by BECK (1939), is used for the isolation of a lipopolysaccharide component from protein antigen. The serological properties of this component are dealt with in this paper.

MATERIALS AND METHODS.

Preparation of the protein antigen.

The protein antigen was prepared according to the directions given by D'ALESSANDRO and DARDANONI (1953), as previously described (DE BRUIJN, 1957).

Preparation of the lipopolysaccharide component from protein antigen.

To 15 ml of the antigen, 5 mg trypsin (B.D.H.) and 0.15 ml of a 1% 'Merthiolate' solution were added. The pH was adjusted to 8.5 by the addition of 0.1 N sodium hydroxide solution and the mixture incubated at 37°C. for 4 days. The pH was checked daily and, if necessary, readjusted to 8.5. When half the incubation time had elapsed, a second dose of 5 mg trypsin was added.

The mixture was centrifuged, the sediment discarded and 40.5 ml absolute ethanol added to the supernatant fluid, thus giving a concentration of 68 % ethanol by weight. The resulting precipitate was allowed to settle at 5°C. overnight, separated by centrifugation and dissolved in 15 ml 0.85 % sodium chloride solution.

Preparation of the anti-protein-antigen rabbit serum.

For the immunization a rabbit was chosen whose serum was initially non-reactive when tested with the antigens in dilutions equal to those which were used afterwards.

The antiserum was prepared by means of intravenous injections of 0.1, 0.2, 0.3, 0.4 and 0.5 ml of protein antigen on the 1st, 4th, 7th, 10th and 13th day respectively. On the 18th day the rabbit was bled by heart-puncture.

The serum was heated at 61°C. for 30 minutes prior to being tested.

Complement fixation test.

The CFT used was a highly standardized routine technique

(DE BRUIJN, 1958a). The syphilitic serum was a pool of human sera. Appropriate serum and antigen controls were included in each test.

RESULTS AND DISCUSSION.

In our previous communication (DE BRUIJN, 1958b), the reactivity of protein antigen with human syphilitic serum was shown to be caused by a major (protein) and a minor (lipoidal) component. Both these components are inactivated when protein antigen is heated in boiling water for 30 minutes (table 1). Anti-protein-antigen rabbit serum, however, still reacts with the heated antigen (table 2). In this case a third antigen-antibody system, which has its optimum in the same region as that of the protein component, seems to play a part.

TABLE 1.

Titration of syphilitic serum with unheated and heated protein antigen.

Serum dilutions	Unheated antigen dilutions						
	1 : 2.5	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
1 : 5	++++	++++	++++	++++	++++	++++	++++
1 : 10	++	+++	++++	++++	++++	++++	+++
1 : 20	—	—	—	+	++	+++	++
1 : 40	—	—	—	—	—	—	—
Serum dilutions	Heated antigen dilutions						
	1 : 2.5	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160
1 : 5	—	—	—	—	—	—	—

From the heat-stability of the antigen, its polysaccharide nature was assumed. To prove this assumption, the method of RAISTRICK and TOPLEY (1934) for the isolation of lipopolysaccharide antigens from bacteria was applied to protein antigen. The isolated component did indeed show a pattern resembling that of heated protein antigen in its reaction with anti-protein-antigen serum. Heating this component in boiling water for 30 minutes did not alter the optimum in the higher dilutions, which can be attributed to the reactivity of its polysaccharide part (table 3).

In the lower dilutions, however, a heat-labile antigenic factor seems to be active which can presumably be identified with the lipoidal part of the isolated component. The lipopolysaccharide was found to be inactive with human syphilitic serum. Since, moreover,

TABLE 2.

Titration of anti-protein-antigen serum with unheated and heated protein antigen.

Serum dilutions	Unheated antigen dilutions									
	1 : 2.5	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
1 : 5	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++
1 : 10	++++	++++	++++	++++	++++	++++	++++	++++	++++	+++
1 : 20	++++	++++	++++	++++	++++	++++	++++	++++	++++	++
1 : 40	++++	++++	++++	++++	++++	++++	++++	++++	++	—
1 : 80	—	—	++	+++	++++	++++	++++	++	—	—
1 : 160	—	—	—	—	—	—	—	—	—	—

Serum dilutions	Heated antigen dilutions									
	1 : 2.5	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
1 : 5	+++	+++	++++	++++	++++	++++	++++	++++	+++	+
1 : 10	—	+	+++	++++	++++	++++	++++	++++	+++	+
1 : 20	—	—	—	+	+++	++++	++++	++	+	—
1 : 40	—	—	—	—	—	—	—	—	—	—

TABLE 3.

Titration of anti-protein-antigen serum with the unheated and heated lipopolysaccharide component.

Serum dilutions	Unheated antigen dilutions									
	1 : 2.5	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
1 : 5	++++	++++	++++	++++	++++	++++	++++	++++	++++	+
1 : 10	++++	++++	++++	++++	++++	++++	++++	++++	++++	+
1 : 20	—	—	—	+	++	+++	++++	++++	+++	+
1 : 40	—	—	—	—	—	—	—	—	—	—

Serum dilutions	Heated antigen dilutions									
	1 : 2.5	1 : 5	1 : 10	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1280
1 : 5	+	+	++++	++++	++++	++++	++++	++++	++++	+
1 : 10	—	—	—	++	++++	++++	++++	++++	++++	+
1 : 20	—	—	—	—	—	+++	++++	++++	+++	+
1 : 40	—	—	—	—	—	—	—	—	—	—

it is isolated from the alcohol-insoluble part of the protein fraction, its lipid cannot be identified with the 'ubiquitous lipid'. Although this points needs further investigation, it is suggested that the lipoidal part of the isolated component may be identical with the fraction, serologically related to alcoholic extract of beef brain and isolated from the Reiter treponema by D'ALESSANDRO, ODDO, COMES and DARDANONI (1949).

The absence of an antibody against Reiter polysaccharide in human syphilitic serum and its presence in anti-protein-antigen serum may point to the fact that one of the differences between a virulent *Treponema pallidum* and the avirulent Reiter strain is localized in the polysaccharide fraction.

Summary.

From the results of immunochemical studies it is concluded that the protein fraction of the Reiter strain of *Treponema pallidum* is a lipopolysaccharide-protein complex. The polysaccharide part of the complex does not interfere with the use of protein antigen in the serodiagnosis of syphilis, because there is no corresponding antibody in syphilitic serum.

Acknowledgement.

The author is indebted to Miss J. H. L. DE JONG and Mr S. DE BRUIJN for technical assistance.

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(From the National Institute of Public Health, Utrecht, Netherlands).

ON THE STABILITY OF REDUCED FLUID STREPTOLYSIN O

by

J. G. HEYL

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One of the most important conditions for reliable determinations of antistreptolysin O in blood in cases of acute rheumatism is the possession of a stable preparation of reduced streptolysin O. In the beginning of the investigations of this part of the serology streptolysin O was freshly prepared and reduced just before use, or stored at + 4°C. in oxidized state and reduced before starting on the antistreptolysin titrations. This method had the disadvantage of time-consuming preliminary work before the determinations could be carried out and often different batches of streptolysin O had to be used. Especially for routine diagnostic laboratories the increase of interest in the serology of rheumatism and the corresponding increase of the number of blood samples received for investigation made it necessary to have a stable preparation of reduced streptolysin O in store.

Kalbak (1947) covered his reduced streptolysin O preparations with sterile fluid paraffin and kept them in the refrigerator at + 4°C. In his opinion the hemolytic property of the streptolysin O would remain constant for about three months.

Van Loghem-Langereis (1950) however, preparing and storing streptolysin O according to the method of Kalbak, observed a decrease of the hemolytic capacity of streptolysin O after three weeks storage at + 4°C. As a result of some experiments on the influence of temperature on the hemolytic stability of stored reduced streptolysin O she introduced the method of keeping these preparations at – 30°C.

Oskam (1958) tried to prevent re-oxidation by adding hydroquinone as an oxido-reduction stabilizer to the reduced streptolysin O. Titrations of these preparations show a lowering of the hemolytic titre after some weeks, even if they have been kept at – 20°C. In our laboratory the best results are obtained by the following method.

Todd-Hewith broth is inoculated with a 6 hour serum broth culture of streptococci (strain S 84) and incubated at 37°C. for 24 hours. It may be said here that, keeping broth-cultures on hand, daily inoculation of the streptococci into fresh serum broth is necessary to keep the hemolytic power at a high level. After incubation the Todd-Hewith broth is sterilised by filtration through a Chamberland L 3 candle; the first 200 ml of filtrate are discarded. To remove air the sterile filtrate is put under vacuum for one hour; then the reducing agent (0.1 % sodium hydrosulphite $Na_2S_2O_4$) and 1 % 1 N NaOH are added and the filtrate exposed to a vacuum for another hour. As quickly as possible the reduced filtrate is divided into smaller volumes and at practically the same time is covered with a layer of about 7 cm of sterile fluid paraffin. The flasks are stored at – 20°C. No batch of streptolysin O is used before a couple of weeks after preparation because, in the first place, during this period any trace of streptolysin S will be removed from the reduced filtrate and, secondly, because, in our experiences, the hemolytic titre of streptolysin O, prepared in this way, increases to a constant level in the first weeks of storage.

In the following table the results are summarized of 10 batches of streptolysin O titrated just after preparation and controlled at different times afterwards.

TABLE 1.

Titrations of the minimum hemolyzing dose (m.h.d.) of stored streptolysin O.

Batch nr.	m.h.d. at preparation	Controlled after	m.h.d.
1	1 : 16	1 week	1 : 64
2	1 : 32	4 weeks	1 : 128
3	1 : 64	7 weeks	1 : 128
4	1 : 32	8 weeks	1 : 128
5	1 : 64	9 weeks	1 : 256
6	1 : 128	10 weeks	1 : 128
7	1 : 128	13 weeks	1 : 512
8	1 : 128	14 weeks	1 : 256
9	1 : 128	16 weeks	1 : 256
10	1 : 128	17 weeks	1 : 256

We may conclude that covering reduced fluid streptolysin O with a thick layer of sterile paraffin and keeping it at low temperature is a reliable and suitable method for having a stable preparation in store for several months, as was already confirmed by van Loghem-Langereis.

Study is in progress on the making of a freeze-dried product as a more suitable form of stored streptolysin O and of a same stability as the fluid preparation.

Summary.

A method for preparing and storing reduced streptolysin O is described. The reliability of this method has been confirmed by the results of the titrations of different batches of reduced streptolysin O at different times after preparation.

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(Research Department, British Vinegars Ltd., Frome, Somerset, England).

A RE-ASSESSMENT OF THE GENUS *ACETOBACTER*

by

J. L. SHIMWELL

(Received October 13, 1958).

INTRODUCTORY.

In 1955 Dr ROBERT S. BREED, Chairman, Board of Editors, Bergeys Manual, in a number of private communications, expressed dissatisfaction with existing classifications of the acetic acid bacteria, and urged me to carry out the work necessary for a re-assessment of the genus and for the clarification of its taxonomy, and to regard this as a "major project" in this research department. This suggestion was approved by the Directors of this Company, and the work was accordingly embarked upon. In this connection the results of some 5 years' previous personal study of these bacteria were available to be drawn upon at the outset. Owing also to FRATEUR's excellent monograph on the genus *Acetobacter* (1950), and to LEIFSON's more recent (1954) discovery that the acetic acid bacteria are not a homogeneous group (either morphologically or biochemically), the work has been greatly facilitated and accelerated.

However, the unexpected discovery, in this laboratory, that almost all *Acetobacter* species and strains undergo extraordinarily facile mutation, whereby mutants are produced which are indistinguishable from other existing classical *Acetobacter* species, or constitute "new" species, has thrown an entirely new light on the whole subject, and has led to the inescapable conclusion that strains of the genus *Acetobacter* proper (as distinct from *Acetomonas* Leifson) cannot be classified at all, no matter what criteria are selected for the purpose.

DEFINITIONS.

The following definitions hold good throughout the ensuing paper:

"*Acetobacter*". This is used as re-defined by LEIFSON (1954) to include only those acetic acid bacteria which oxidize ethyl alcohol through acetic acid to CO_2 and H_2O. In motile strains of this genus the flagella are peritrichous (not polar, as previously wrongly stated in the literature).

"*Acetomonas*". This is used as defined by LEIFSON (1954), and amended by SHIMWELL (1958), to denote those bacteria oxidizing alcohol only as far as acetic acid (these in FRATEUR's (1950) classification, fall into his "suboxydans group"). When motile they possess one or more polar flagella.

"Species". Except when otherwise stated, the use of a specific name means that the diagnosis is that of FRATEUR (1950), and that his methods were used for identification.

"Mutation". This term is used in the widest possible sense to denote all heritable changes of characteristics; true genic mutation in *Acetobacter* cannot be distinguished from possible heritable changes of characteristics due to cytoplasmic phenomena except by segregational analysis, which has not yet been attempted in this genus.

"Ketogenic". This term is used solely to denote the production of dihydroxyacetone from glycerol, as this is really the sense in which it is used by FRATEUR (1950). The possible production of 2-ketogluconic acid, as distinct from 5-keto acid, by otherwise "non-ketogenic" species is not used by him in his classification.

Colony photographs. All the colonies illustrated were those obtained on unhopped beer-agar (pH 6.0) after 48 hours at 27°C. Magnification × 40.

EXPERIMENTAL.

The criteria available for the classification of saprophytic bacteria fall into 3 types, (a) morphological, (b) cultural, and (c) biochemical.

(a) **Morphological.** The pleomorphism of the acetic acid bacteria has long been cited in text-books as a classical example of this phenomenon, and HANSEN's original illustrations of long swollen filaments, and other "involution forms", still appear from time to time in text-books of bacteriology. No particular significance has apparently been attached to the prevalence of such bizarre cell forms, however; even in the 'twenties and 'thirties, when exaggerated claims regarding so-called "life-cycles" were sometimes being made, the pronounced pleomorphism found in *Acetobacter* was scarcely mentioned.

In the course of the present study the examination of a large number of strains of all available classical *Acetobacter* species has confirmed the prevalence of such pleomorphic cell forms in many species or strains. Apart from these "abnormal" forms the cell-shape and size of different species has ranged from almost coccoid cells on the one hand to long straight or curved rods on the other. In particular the prevalence of curved crescent-shaped pairs of cells has been a notable feature of some species. However, it was only in few cases that cell-form showed correlation with cultural and biochemical characters. Moreover it was frequently found that two or more cell-forms (e.g. coccoid and crescent-shaped) occurred in one and the same culture, even when young. Cell-shape and size, therefore, has afforded no means of differentiating species, except sometimes, perhaps, in the case of *A. xylinum*, where the production of a thick leathery pellicle giving "the cellulose reaction" was often correlated with the crescent-shaped cell-pairs previously mentioned. As, however, celluloseless mutants of *A. xylinum*, corresponding to *A. mesoxydans* Frateur, have also shown crescent-shaped pairs, this criterion has proved of little value (Fig. 1). But although morphology, therefore, has not been found of value for species differentiation, the possible significance of certain swollen forms will be referred to later under "Mutation" etc.

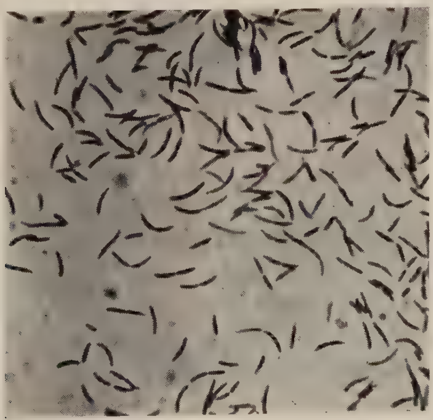


Fig. 1. *A. mesoxydans* strain. Crystal violet. × 1000.

(b) **Cultural Characteristics.** These consist of the nature of growths on liquid and solid media. The liquid media traditionally used often contain ethyl alcohol as main carbon or energy source. Such media would seem to be essentially artificial, however, as it is difficult to conceive any natural habitat where a quiescent liquid medium, free from jarring or ruffling of the surface, could occur, (let alone one containing ethyl alcohol). Yet those are the conditions required, in the laboratory, for the formation of a surface film by *Acetobacter* strains. A number of such species can be isolated from natural habitats such as soil, vegetable matter, bruised fruit, and the gummy exudates of certain trees, but the majority of species recorded in the literature were isolated from such artificial habitats as beer, wine, cider, and vinegars of various types. From what will be recorded later it seems probable that most of such species (and therefore most of all named *Acetobacter* species) may be mutants of naturally occurring types, selected by the artificial environments from which they were eventually obtained.

Apart from the production of the characteristic thick leathery film by *A. xylinum*, which largely defines this species, the other types of film produced have varied from thickly viscous through dry and coherent to friable and fragmentary, and even to no film at all (when the cells grew slowly as a submerged haze on dissolved oxygen). No reliable correlation has been found between type of film, cell-shape and size, and biochemical activities.

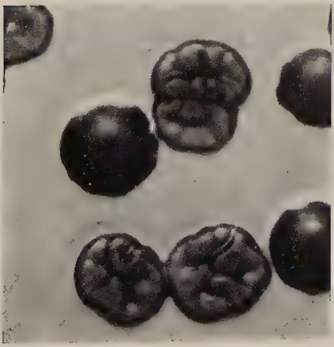
On solid media the study of cultural characters involves the observation of the appearance of (1) growths on the surface of agar or gelatin slants or plates and (2) of separate colonies resulting from smearing a dilution of the bacteria on the surface of plates of the same media. The appearance of streak cultures on the surface of solid media, however, is really only that of innumerable confluent colonies, and has shown much less variety of form than has the study of the appearance of isolated colonies themselves.

The texture of streak cultures on various media has varied greatly from strain to strain, but (except perhaps in the case of *A. xylinum* again) has not been found to be consistently correlated either with cell-form or with biochemical properties. Thus viscid growths have proved to be either cellulosic, or starchy, or of some other polysaccharide giving neither reaction (Shimwell and Carr, 1958). In the case of *A. xylinum*, however, the surface growth has usually been papery or very coherent.

Separate colonies of different *Acetobacter* species or strains, however, have often shown great diversity of appearance. By reflected light, particularly with the naked eye, such differences in form may escape notice, the colonies often appearing relatively featureless, of circular, or roughly circular, shape; white, sometimes pink in colour, flat, raised, or convex, and matt to glistening in appearance. Cellulosic (and to a lesser extent starchy) strains have usually yielded smaller and more slowly growing colonies than others.

It was only when one came to examine such various colonies by oblique transmitted light, with a magnification of × 40, using a × 4 objective and a × 10 eyepiece (not the reverse combination) that the amazing diversity of shape, opacity, contour, and structure appeared so strikingly (Shimwell 1956, 1957a). It is this method of examination which has led to the detection, in pure cultures, whether obtained from culture collections or freshly isolated in this laboratory, of mutants corresponding to other known species, or constituting previously undescribed species.

Although colonies differing from the majority of those on a plate have often been found to differ in biochemical properties and cell-form from those of which the bulk of the cultures consisted, no consistent correlation with such other properties has been found, except in the case of small, irregularly circular, almost opaque, dented or wrinkled colonies (Fig. 2), which have always, so far, indicated *A. xylinum*. The converse of this, however, did not apply; all cultures giving a thick leathery cellulosic pellicle on liquid media did not necessarily all produce colonies of the form described and illustrated. Some such cultures yielded perfectly circular, smooth, opaque colonies, whilst other were relatively translucent, rough or limpet-like in shape (Fig. 3).



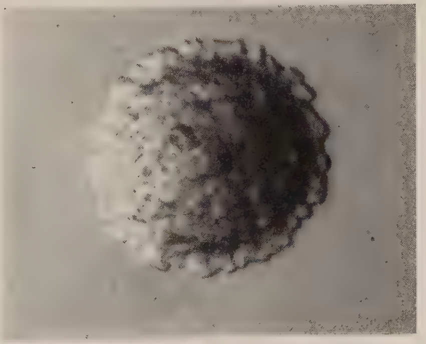


Fig. 2. "Typical" wrinkled *A. xylinum* colonies. (Also mutant).
Fig. 3. "Rough" *A. xylinum* colony.

Colony Mutants. Mutations involving drastic alteration in the type of colony produced, but not simultaneously associated with changes in FRATEUR's biochemical properties, have been numerous.

In particular, the case of *A. lovaniense* N.C.I.B. 8620 has been outstanding. It has been previously recorded elsewhere (SHIMWELL 1957*a*) that this was the only *Acetobacter* strain then studied which proved completely stable, no mutants involving either change of colony form or biochemical properties being detectable. After maintaining this strain for 2 years in chalk-wort media by THAYSEN's method (1924) a complete change of colony form has occurred. Instead of the rather beautiful raised rugged colonies produced by the original N.C.I.B. culture (Fig. 4), these have become entirely replaced by a mixture of two different colony forms, which could perhaps be described as (a) poached-egg, and (b) bun-like (Fig. 5).

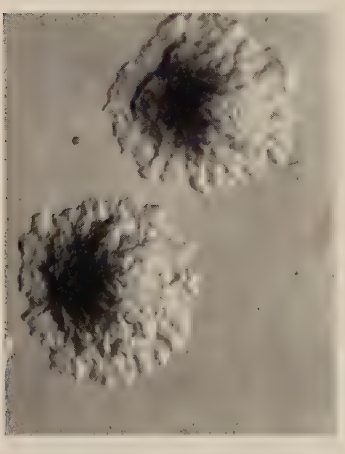


Fig. 4. *A. lovaniense* original colonies.

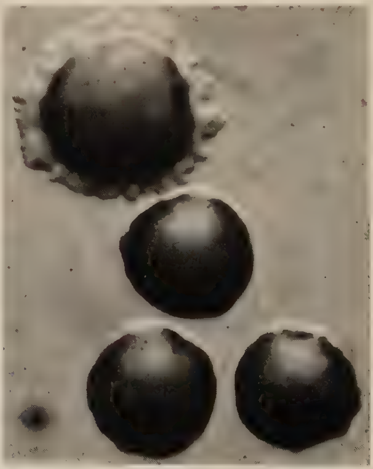


Fig. 5. *A. lovaniense* mutant colonies.

Neither of these new colonies, however, on being separately picked and cultivated, showed any biochemical difference from the original N.C.I.B. culture. In order to ensure that the changed colony form was not due to plating on a new batch of solid media (unhopped beer-agar) another freeze-dried ampoule of the same strain was obtained and plated on the new medium, whereupon all the colonies produced were of the original type (as in Fig. 4).

Similar changes of colony form, independent of biochemical character, have been obtained with a number of *Acetobacter* species.

(c) **Biochemical Characters.** FRATEUR (1950) had, of course, previously formed a somewhat similar opinion of the inadequacy of morphological and cultural characteristics, and preferred biochemical criteria, selecting 5 of these as the basis of his classification and method of species identification because he considered that they showed "remarkable stability." These criteria were (1) catalase, (2) ketogenic power, (3) the production of acid from glucose, (4) growth on Hoyer's medium, and (5) the production of a thick leathery film giving the cellulose reaction.

Applying these criteria to a collection comprising strains of all recognised and available species of *Acetobacter*, and also many new isolates from various sources and habitats, FRATEUR constructed an attractive, simple, and rapid method of species identification based on an equally simple and logical classification. Eight species of *Acetobacter* proper were recognised (although FRATEUR emphasized that his work was incomplete). This system has been tested, approved, and acclaimed by a number of workers as affording, at last, a logical and practicable method of classifying and identifying newly isolated *Acetobacter* strains, and has proved to be of great value in this laboratory.

It is therefore with some regret that it now has to be recorded that the apparent stability of the biochemical properties on which the system is based is illusory – unless one makes the arbitrary assumption that an *Acetobacter* culture is, and remains, biochemically homogeneous, whereas it has been found, in this laboratory, that the majority of *Acetobacter* cultures are, or soon become, mixtures of two or more "species" derived by spontaneous mutation, and that these can be readily separated by simple surface plating, picking off individual colonies of different form, and transferring them to separate agar slopes (also SHIMWELL 1956, 1957a).

In the last two years a larger number of *Acetobacter* strains, some from culture collections, others freshly isolated from beer, vinegar, and cider, have been studied, and again it has been found that such strains, even after rigorous "purification" by plating and replating, have, in the majority of cases, rapidly given rise to spontaneous mutants which have lost or gained some biochemical property or other, this shifting them into some other species and often even involving a move into another of FRATEUR's groups.

A full account of all the results and experimental work involved would be too lengthy for inclusion in this paper; it is hoped, however, to incorporate this in a further communication in due course. In the meantime the following examples will serve to illustrate the type of phenomena encountered, and its profound implications concerning the taxonomy of the *Acetobacter* genus:

1. An isolate identified as of the species *A. xylinum*, using FRATEUR's criteria, and apparently stable over a long period of serial transfers, has then been found to yield, upon plating, 3 distinct and different colony types, (1) opaque dented, (2) rough translucent, and (3) smooth, circular, semi-opaque. These, upon picking and separate cultivation, proved to be respectively (1) *A. xylinum* (FRATEUR's diagnosis), (2) *A. mesoxydans*, and (3) *A. rancens*. Table 1 shows the biochemical properties of these three "species".

TABLE 1.

	A. xylinum	*A. mesoxydans*	*A. rancens*
Catalase	+	+	+
Hoyer's Medium	—	—	—
Ketogenic power	+	+	—
Acid from glucose	+	+	+
Cellulose	+	—	—

It will be seen that as all the positive biochemical properties of *A. mesoxydans* and *A. rancens* are included amongst those possessed by *A. xylinum*, the culture itself can only be identified as *A. xylinum*, although only a proportion of the cells in the culture are of that species.

The above *A. mesoxydans*-like mutant, after quite a relatively short period of separate serial transfer, was found to give rise to a mutant corresponding to *A. rancens*, the presence of the latter species in the original *A. xylinum* culture being thus explained. Similarly, the new "purified" *A. xylinum* single colony culture soon gave rise to cells of *A. mesoxydans*, the presence of the latter in the original culture being thus accounted for.

2. *A. pasteurianum* gave rise to a starchless mutant indistinguishable from *A. rancens*, this mutation being irreversible (SHIMWELL 1957b).

(Frateur regards this species as a starch-positive variety of *A. rancens* because starch-production is notoriously so easily lost).

3. *A. mesoxydans*, by gain of cellulose production, yielded *A. xylinum*.

4. *A. ascendens* gave rise to a mutant which, by loss of catalase, had become *A. paradoxum*.

5. The most striking example of all, however, was provided by a certain culture which, as a whole, reacted as *A. mesoxydans*. This, upon plating after 3 months' weekly serial transfer on beer-agar (pH 6.0), yielded 3 different colony types (Fig. 6), which upon picking and separate cultivation were respectively found to have the biochemical properties shown in Table 2 (The small colonies subsequently developed into one or other of the other 3 types).

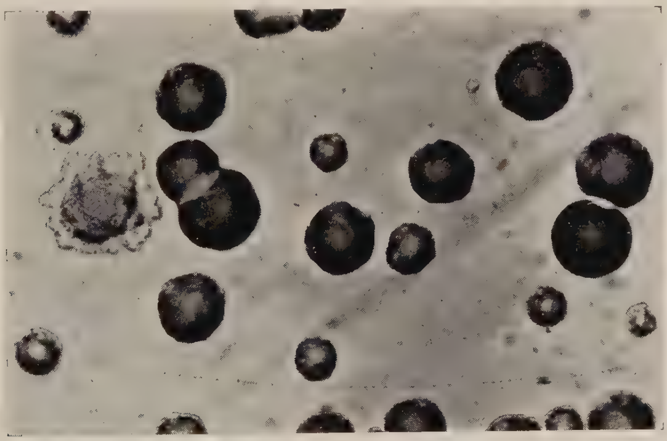


Fig. 6. Colonies of "composite" *A. mesoxydans* strain.

TABLE 2.

Constituent "species" of an "*A. mesoxydans*" culture.

	1	2	3	Reaction of whole culture
Catalase	—	+	—	+
Hoyer's Medium	—	—	—	—
Ketogenic power	—	—	+	+
Acid from glucose	+	+	+	+
Cellulose	—	—	—	—

It will be seen that although the whole culture is indisputably *A. mesoxydans*, according to FRATEUR's criteria, not one of the constituents of that culture is of that species. The characteristics of the culture are merely the "resultant", or sum, of the combined biochemical properties of its constituent cells.

It will also be noted that when one or more of the biochemical properties of a culture-constituent is negative, and where such properties are positive in the case of one or more of the other culture-constituents, then such negative constituent cells do not affect the biochemical species-identification of the culture as a whole. They merely act as diluents, thus reducing the intensity of the particular biochemical reaction of the whole culture. It has been repeatedly found, here, that where a culture yielded a weak, but definite, particular biochemical reaction, this was not due to a feeble possession of that property by all the cells in the culture, but usually to the presence of mutants in which that particular reaction was negative.

Purification of Cultures. From the above it will be seen that the "purification" of a culture, by the usual method of replating and picking well-isolated colonies, is difficult. Indeed, in the case of the *A. mesoxydans* strain referred to above as containing 3 constituent "species", it is impossible; for the picking of any individual colony results, not in the maintenance of the characters of the culture, but in a change of species to one with different characters. Even in less complex cases loss of the original characteristics of the culture may be more probable than their maintenance, particularly if the reason for the purification is that one of the biochemical characters of the culture is waning in intensity. Such waning may well indicate that mutants devoid of that particular property now predominate in the culture. If so, plating and picking is more likely to select a negative strain.

The only methods (other than immediate freeze-drying) which would seem available for the maintenance of the original "species" are:

1. Maintaining the culture on some selective medium known to favour the predominance of cells with the desired characters.

2. Plating and picking numbers of colonies, separate cultures of which are then submitted to all the biochemical tests necessary, the one with the required combination of properties being selected (if it is still there), the others being discarded. Even this method,

however, is not without its snags. It may be found that the selected colony, although it possesses the desired biochemical properties, may differ considerably in some other respect (e.g. culturally) from the original culture. This has, indeed, often happened in the case of *A. mesoxydans* strains obtained from malt vinegar, particularly if such strains have been obtained as celluloseless mutants of an *A. xylinum* strain. Such *A. mesoxydans* strains have often apparently back-mutated, by gain of cellulose-production, to yield *A. xylinum*, but such cellulosic back-mutants are not necessarily of the same genotype as that of the original *A. xylinum* cells from which the *A. mesoxydans*-like mutants were obtained. In many cases the cellulosic pellicle produced on liquid media has lacked the tough, thick, leathery consistency of that produced by the original genotype, tending to be more like tissue-paper, a characteristic reminiscent of HENNEBERG's (1926) description of *A. orleanense* or *A. xylinoides*. Moreover, the colony form may be quite different from that of the original *A. xylinum* strain, being rough and translucent (as in Fig. 3) as distinct from the usual opaque, dented, or wrinkled colonies of so-called typical *A. xylinum* (Fig. 2).

Thus even if purification by replating appears to have resulted in the reclamation of a strain biochemically identical with the original one in respect of FRATEUR's criteria, this strain is not necessarily identical in genotype in respect of other criteria.

New Species. When the facile mutability of *Acetobacter* species first came to light (SHIMWELL 1956, 1957a) it was pointed out that if all 5 of FRATEUR's biochemical criteria should be mutable, then the number of species possible in his classification would be 2^5 (= 32). This prediction is being fulfilled. It is already possible to add a number of new combinations of biochemical properties to the 8 species originally recognised by FRATEUR. The total number of species "discovered" to date, together with their biochemical characteristics is shown in Table 3.

Attention is drawn to the cellulose-positive *A. aceti* strain in the first line. This strain was isolated by CARR (1958b) from cider, and (as CARR himself pointed out) this "new species" has the distinction of having all FRATEUR's biochemical properties positive, thus completing one end of the hypothetical series of 32, the other end being already occupied by *A. paradoxum* (a new species isolated by FRATEUR himself, 1950) in which all such properties are negative. As CARR further remarks, "It would, therefore, only appear to be

TABLE 3.

Acetobacter "species" so far discovered.

(Arranged in descending order of positive biochemical properties)

	No. of properties	Catalase	Hoyer	Ketogenic	Acid from glucose	Cellulose
1. "New Species" A¹)	5	+	+	+	+	+
2. *A. aceti*	4	+	+	+	+	—
3. *A. xylinum*	4	+	—	+	+	+
4. *A. estunense*²)	4	+	+	—	+	+
5. *A. mesoxydans*	3	+	—	+	+	—
6. "New Species" B	3	+	—	—	+	+
7. *A. lovaniense*	3	+	+	—	+	—
8. "New Species" C	3	—	—	+	+	+
9. "New Species" D	2	—	—	+	+	—
10. *A. rancens*	2	+	—	—	+	—
11. *A. ascendens*	1	+	—	—	—	—
12. *A. peroxydans*	1	—	+	—	—	—
13. "New Species" E	1	—	—	—	+	—
14. *A. paradoxum*	0	—	—	—	—	—

¹) CARR (1958a). ²) CARR (1958b).

a matter of time before all the 32 'species' postulated . . . are discovered".¹)

As previously pointed out elsewhere (SHIMWELL 1957a) only 4 of the 5 biochemical criteria concerned have so far proved to be mutable. The outstanding exception is the ability to grow on Hoyer's medium (which indicates the ability to use ethyl alcohol as sole source of carbon with ammonium salts as sole source of nitrogen). This exception is rather surprising, as the production of auxotrophs is a common mutation recorded in the case of bacteria of other genera. Whether this failure to obtain auxotrophs from the 3 known and named Hoyer-positive *Acetobacter* species (*A. peroxydans*, *A. lovaniense*, and *A. aceti*) indicates some fundamental difference

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¹) The recent claims of STEEL and WALKER (1957) that certain N.C.I.B. cultures of *A. xylinum*, *A. xylinoides*, and *A. acetigenum* are motile, and give rise to motile, putrefactive, proteolytic, peptonizing polar-flagellated, *Pseudomonas*-like mutants which have lost all the biochemical properties of the genus *Acetobacter*, and now most closely resemble *Ps. geniculata*, has not been confirmed in a single particular (SHIMWELL and CARR 1958). No mutant possessing even one of the properties possessed by those of STEEL and WALKER has been obtainable from the same parent strains – which were also non-motile.

in, say, the phylogenetic origin of these species as compared with the remaining species, or whether the mutation is a rare one which further work will discover in time, is not yet clear. If the first alternative is correct then the postulation of 32 possible combinations of the 5 biochemical criteria would need appropriate modification. On the other hand the discovery that cellulose-production is virtually as mutable as starch-production would logically require that the exclusion of starch-production from amongst the criteria used for species differentiation should be reconsidered, particularly as extracellular starch-production is rare amongst other bacteria.

If it is added to the list of such criteria then the number of possible species is forthwith doubled, and 64 species become possible.

As also there are those who do not care for species to be established on solely biochemical grounds, but consider that morphological and cultural considerations should also be taken into account, it is perhaps worth further emphasizing that each character added to the list of species criteria will, if it is mutable, again double the total number possible [and, indeed, morphological and cultural characters do seem just as mutable as are biochemical ones (SHIMWELL 1956, 1957a)].

It is also pointed out that, in the foregoing, only 5 biochemical criteria have been considered, these having been selected by FRATEUR partly on account of the relative ease with which the possession or absence of such properties can be determined in the laboratory. There are, however, many other less easily determinable biochemical processes carried out by *Acetobacter* species, and, apart from ease of laboratory detection, it could be argued that some are just as logical and fundamental for species differentiation as are FRATEUR's 5 selected criteria. Indeed, many carefully purified cultures of *Acetobacter* strains give rise to a variety of colonies which do not differ from one another in respect of the 5 criteria mentioned, e.g. *A. aceti* (SHIMWELL 1957a), but it is not improbable that they may differ in respect of other biochemical properties. It also scarcely needs mentioning that additional consideration of sugar-fermentations would multiply the number of possible combinations to an astronomical magnitude and would result in a "pulverisation of species" even greater than that envisaged by BEIJERINCK.

In addition to the phenomena described in the foregoing, which nearly all refer to observations made in this laboratory, FRATEUR and SIMONART (1952), studying the flora of an industrial acetifier producing spirit vinegar of high acidity (11% acetic acid w/v), have reported the occurrence, towards the end of the acetification, of increasingly numerous bacteria yielding dwarf colonies on conventional media. These "dwarf mutants" showed exceptional resistance to the high acidity, this accounting for their predominance at the end of the process.

Some of these dwarf mutants were identified, and found to be, apart from their dwarf-colony nature, of the species *A. rancens*, *A. mesoxydans*, and *A. xylinum*, thus not being considered to be new species or varieties but mutants of existing classical species. These authors also isolated, at different stages in the acetification, strains corresponding biochemically to *A. rancens* and *A. mesoxydans* except that they were catalase negative. The taxonomic position of these was left open.

Wiame, Dothey, and Harpigny (1957) have also reported the isolation, from an experimental acetifier, of an acetic acid bacterium which only grew in very acid media, growing well at pH 3.0 but not at pH 4.5. They provisionally named it *Acetobacter acidophilum*. It could not be isolated except on very acid media, and the presence of CO_2 was imperative.

From these examples it will be seen that, quite apart from the conventional criteria used for the classification and identification of species, there may be many others contributing to the multitude of ways in which one *Acetobacter* strain can differ from another, or even from one of its own mutants. Perhaps the most fundamentally important of these have yet to be discovered.

Discussion.

From the foregoing it is submitted that the classification and identification of *Acetobacter* strains is virtually impossible. All the experience gained in this laboratory has shown that no sooner has one "identified" an isolate than it usually begins to change its biochemical or other characteristics. In some cases the culture as whole may retain its distinguishing characters over fairly long periods of sub-cultivation, but even then it will often be found that one property or another will begin to wane until it has perhaps been lost altogether. Sometimes a culture will gain some biochemical or other property of another species. In either case many cultures of *Acetobacter* will eventually cease to correspond to the species they were originally found to be.

Even in those cases where the culture, as a whole, succeeds in retaining all the properties it originally possessed it will soon be found, more often than not, to consist of a mixture of two or more other *Acetobacter* species. "Identification" only shows, therefore, to what species the cells in a culture correspond at the moment of isolation. Within a short time (sometimes in only a matter of weeks) such identification may be no longer correct.

Some properties are more easily lost or gained than others. Ketogenic power appears to be one of those most easily lost, as are also starch and cellulose production. Cellulose production is also one of the properties most easily gained (notably by *A. mesoxydans*). As already mentioned, growth on Hoyer's medium is at the other extreme, being highly stable, and indeed, its loss not yet having been detected at all. It has also been noticed that when once a culture, by loss of properties, has become *A. rancens*, a sort of cul-de-sac seems to have been reached, and further loss of biochemical properties is comparatively rare. This may explain why this species is of such common occurrence, and of so many cultural "varieties", being hypothetically derivable, by a single-step mutation, from 5 other species (see Table 3).

It will also be seen that the fewer positive biochemical properties possessed by a strain, the fewer it has to lose.

The extreme case of this is *A. paradoxum* which has lost them all, and is therefore incapable of further mutation, in respect of FRATEUR's criteria, except by regaining some property. *A. paradoxum*, however, upon plating does show more than one colony form (Fig. 7); it would be interesting to know whether such different colony forms are associated with loss or gain of some biochemical property other than one of FRATEUR's five.

Finally it should be mentioned that, when a mutant has been obtained from a certain originally pure culture, this is usually by no means the end of the matter. Such mutants almost invariably give rise to further mutants, and these in turn to still others. For this reason it has been found necessary to discard most of the cultures obtained by separately picking such mutant colonies, as the time was not available for their maintenance, and in any case they usually soon changed into something else. Most of the "species" listed in Table 3, however, are available.

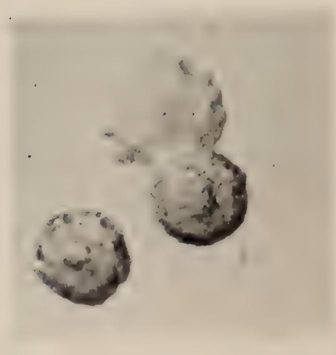

Fig. 7. *A. paradoxum* colonies.

"Mutation", "Cytoplasmic Variation", or "Life Cycle"? Although the term "mutation", for the sake of simplicity, has been employed throughout this paper, some may consider that the phenomena described smack more of cytoplasmic variation, and this may well be the case. Also the possibility of segregation and recombination cannot be excluded. There has been some slight evidence, however, that sometimes a more complex type of reproduction may be involved.

These days one hesitates to mention the term "life-cycle", but observation of some of the large swollen cells, so prevalent, for example, in some strains of *A. mesoxydans* (itself one of the most mutable of *Acetobacter* species) have certainly suggested some phenomenon far removed from multiplication by simple fission.

These swollen cells, many times larger than those constituting the bulk of the culture, usually occurred in groups or clusters, and were found in cultures incubated for as short a period as 48 hours or so. Upon negative-staining (without fixing) a little of the growth from a beer-agar slope, many of these swollen cells appeared to contain a mass of small spherical to rod-shaped bodies. A notable feature of such negative-stained preparations was that in the immediate vicinity of such clusters of large bodies there were, almost invariably, numbers of similar small rods, free, and outside the large bodies themselves, rather suggesting that they had been liberated by the rupture of one of these large bags of rodlets, an explanation which is, of course, purely speculative. Positive staining by various methods and dyes failed to show these small rods. Furthermore, upon plating the culture on beer-agar, yeast-extract-glucose-agar and similar orthodox media, no dwarf colonies suggestive of "L-forms" or similar phenomena were observed, those colonies developing being of normal size, although these included some of different form, found to be biochemically different from the whole culture itself. There is no justification, therefore, for advancing any speculative interpretation of the phenomenon observed; a good photomicrograph was obtained, however, and it is hoped that the loss of definition unavoidably involved in publication will not entirely prevent the minute bodies, within and outside the large cells, from being seen (Fig. 8). A contact print will gladly be sent, on request, to anyone interested.

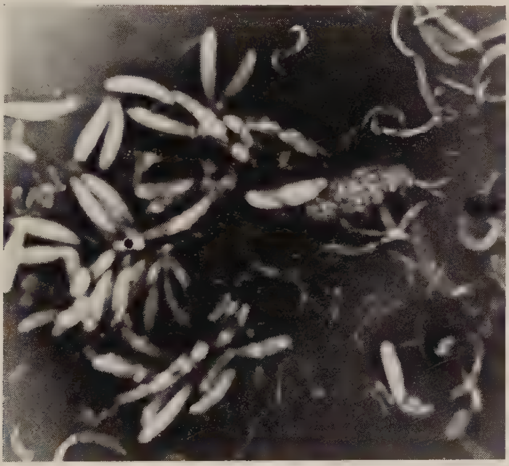

Fig. 8. *A. mesoxydans* strain. Large bodies and small rods. × 1400.

During the work recorded in this paper the feeling has been engendered that the kaleidoscopic changes in the characteristics of *Acetobacter* cultures was rather too facile and frequent to be fully attributable solely to genic mutation, and it may well be that there is some other more complex mechanism involved to which the observation mentioned above may be a clue.

Summary.

An eight years' study of many strains of all available classical species of *Acetobacter* proper (excluding *Acetomonas* Leifson), and many new ones isolated from various materials, has led to the

conclusion that *Acetobacter* strains cannot be classified at all, no matter what criteria are used.

Nearly all strains examined have readily given rise to spontaneous mutants which have lost or gained some major characteristic, thereby becoming indistinguishable from other classical *Acetobacter* species, or constituting "new" species; these, in turn, have given rise to further mutants and so on.

All mutants obtained, so far, have remained within the genus *Acetobacter*, and all motile ones examined have possessed peritrichous flagella, (confirming LEIFSON's findings), the recent claims of STEEL and WALKER (1957) to have obtained polar-flagellated, proteolytic mutants which have lost the power of oxidizing alcohol, and most closely resemble *Pseudomonas geniculata*, not having been confirmed in a single particular.

Some slight evidence has been obtained that the classical pleomorphism in *Acetobacter* may indicate a method of reproduction other than simple fission.

ADDENDUM.

Since the above paper was written, Dr JAMES M. SHEWAN, of Torry Research Station, Aberdeen, Scotland, has generously given me permission to publish an electron micrograph, Fig. 9 (the first ever taken of an *Acetobacter* strain) this being the *A. rancens* strain of which the peritrichous flagella have been previously photographically demonstrated by staining (SHIMWELL, 1958). The further maintenance of *Acetobacter* in *Pseudomonadaceae* is now "indefensible".

Acknowledgements.

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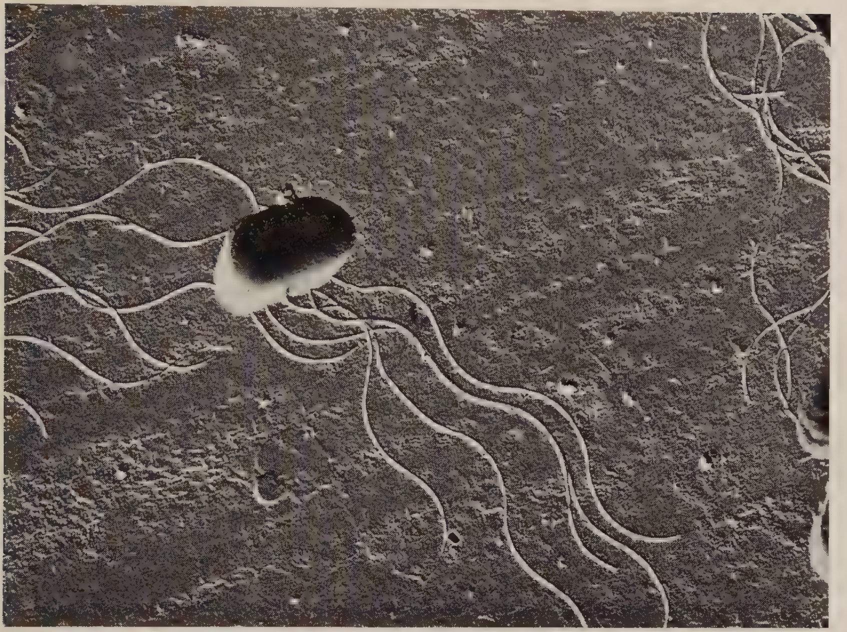

Fig. 9. Electron micrograph of an *A. rancens* strain. × 15,000.

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DISEASES OF THE LARVAE OF *PRODENIA LITURA*

by

B. FLASCHENTRÄGER † and **H. T. SOLIMAN**

(Received June 30, 1958).

Whilst breeding the larvae of *Prodenia litura*, the so-called "cotton-leaf worm", in the laboratory during the years 1947—1951, we found that they were liable to three types of disease. The first was characterized by the appearance of an orange or pink colour on the ventral surface of the prolegs. The larvae, which were mostly 3—4 cm long seemed to be less active under the leaves and crept away at the wall of the net, to remain hanging by the posterior end until they died. If the pink colour was seen, death could be predicted within 2—4 days.

The pinkish pigment was not soluble in ether and its nature is unknown. In the course of the disease the larvae swelled up with the development of gas in the intestinal tract. After death they were completely decomposed inside to a dark, bad smelling liquid. The skin was very weak and ruptured at the slightest touch. It seemed that bacterial decomposition occurred with strongly hydrolyzing enzymes. Dr RUKSHY has isolated some of the bacteria. He believes that *Bacillus proteus* was the cause of this liquefaction. He also found *Staphylococcus aureus*. The blood of the affected larvae was found to contain the polyhedra virus and the symptoms mentioned are identical to those described for the polyhedra virus disease by SALAH ABOUL-NASR (1956). Specimens of dried larvae, pupae and moths were sent to Dr G. BERGOLD in Canada who confirmed the presence of polyhedra bodies, isolated the virus and named it *Borellina litura*.

The second type of disease was observed when the larvae were found scraping over the food without eating it, so that the body gradually shrank. The skin became more and more depressed in rings

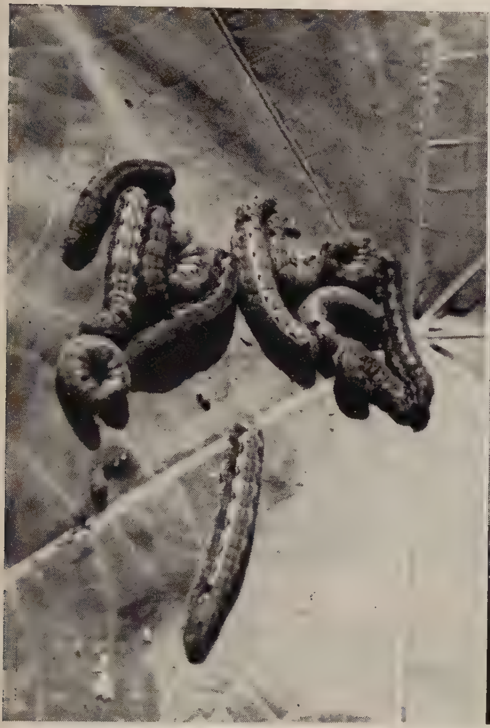

Fig. 1. Healthy larvae on *Ricunus* leaf.

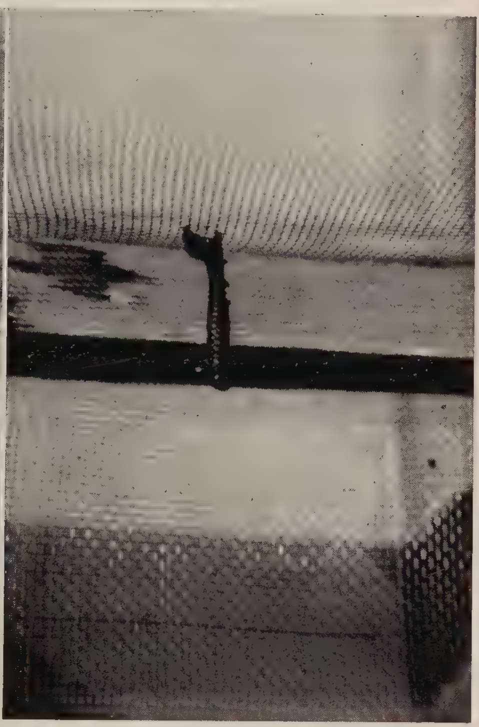

Fig. 2. Diseased larva.

between the body segments and was very rigid, rough and with dry surface. After death the larvae became brown in colour and contained a sticky, bad-smelling material. No reference to a disease of this type could be found in the literature.

The third type of disease was characterized by the complete blackening and mummification of the skin after the fifth instar. The larvae were seen to creep over the food without eating and generally died in 4—5 days. Some developed into adults but very often death occurred in the pupal state or during emergence. In every case the body decomposed internally into a brown, strong smelling liquid.

The diseases of the cotton-leaf worm do not seem to have been previously investigated by microbiologists. Certain bacteria are normally present in the intestinal tract of larvae, living symbiotically and probably aiding in the digestion of food or vitamin synthesis. No information could be found on the presence of fungi in larvae and a systematic investigation was made on the mycological flora of the dead larvae. The fungi were cultivated by one of us (Soliman) on peptone dextrose agar of the following composition: 2 g KH_2PO_4, 0.5 g $MgSO_4$, 4 g peptone, 15 g agar, 20 g dextrose, 1000 ml water.

The following fungi were isolated from the digestive tracts of the dead larvae: *Aspergillus flavus*, *Syncephalastrum*, *Aspergillus niger*, and *Cladosporium*.

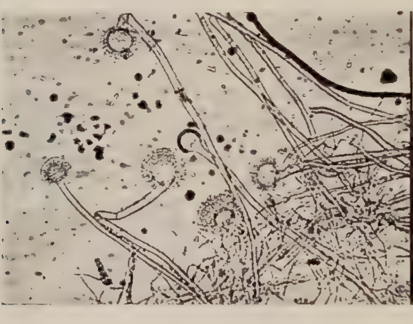

Fig. 3. *Aspergillus flavus*.

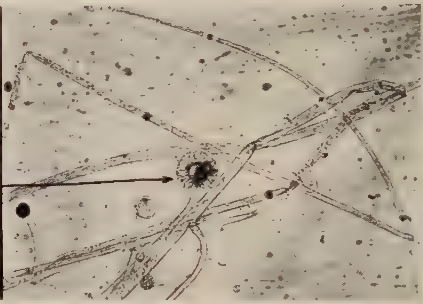

Fig. 4. *Syncephalastrum* spec.

Aspergillus flavus was reported by Burrows as the cause of disease in the external-ear of man. The two latter fungi were found outside the place of inoculation and it seems likely that they were contaminants.

A further species of *Aspergillus* was isolated which was bright yellow in colour. The cells of the hyphae were swollen and flask-shaped but the isolate remained in the vegetative state until the medium had dried out when long conidiophores together with sterile vertical hyphae were produced. The isolate was sent to the Centraalbureau voor Schimmelcultures, Baarn, Holland for identification, and it appears to be a new species which will be described in a separate paper. This fungus was found to have an inhibitory effect on the growth of *Trichoderma* spec.

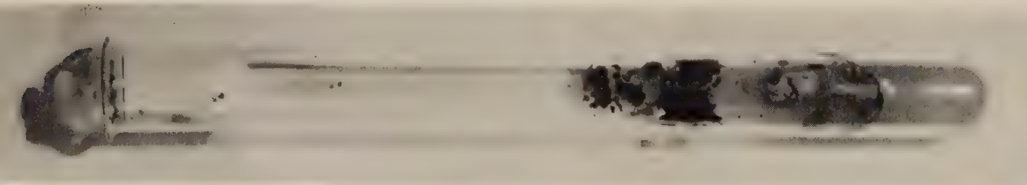

Fig. 5. Inhibitory activity of a yellow *Aspergillus* on *Trichoderma* spec.

An experiment was carried out to investigate the effect of these fungi on healthy larvae on *Ricinus* leaves. The leaves were contaminated with the spores of *Aspergillus flavus* and after feeding for some time the larvae became weak and sometimes died as prepupae.

When it was found that a prepupa of a healthy larva could not complete its life cycle and died, it was sterilised and dissected, after which different parts of its intestinal tract were cultivated.

The larvae were externally disinfected with the following disinfectant: sublimate 2% 500 ml — ethanol 50% 500 ml — hydrochloric acid 37% 20 ml — distilled water 500 ml (SPIRTES, 1936). This mixture was boiled to sterilise the glassware. In order to sterilise the larvae they were placed for 10 minutes in a crucible with 10 ml of the sterilising liquid. The liquid was changed once, and replaced with sterile water to remove any surplus. Physiological 0.9% sodiumchloride solution was preferable to water. A diseased larva could be kept in a tube with sterile 0.9% NaCl without liquefaction after sterilising.

In the cultures a *Penicillium* was found to grow from every inoculum. On a *Ricinus* leaf a reddish fungus was observed; this was also cultivated and here too a *Penicillium* species was found to grow. When a diseased larva with a red abdomen was touched, a green liquid was vomited from which again the *Penicillium* could be grown. When liquefied larvae were cultivated *Candida albicans* and a *Bacillus* species were obtained. From healthy larvae such fungi as *Aspergillus niger*, *Mucor* and *Fusarium* were grown, which were also found, when the gut and the first part of the stomach of larvae fed on *Ricinus* leaves were cultivated. In the other parts of the intestines, however, the fungi were not present.

On examination of the faeces of diseased larvae some spirals and some Penicillia were found. The spirals were recognized to be the thickenings of the xylem elements of the leaf mid-rib and were not found in the faeces of healthy larvae. Thus we could conclude that *Prodenia litura*, when healthy, can digest and destroy fungi, thereby releasing some fungal enzymes which are not secreted by the worm and which can digest otherwise indigestable xylem components.

Bacteria: In case of healthy larvae comma-shaped bacteria and Streptococci were cultivated from the gut and first part of the stomach. From the last part (last 1/3) of the stomach bacilli and Staphylococci were grown in relative very small numbers compared with the comma- and spherical bacteria. The rectum is similar to the gut in its bacterial contents, but the middle part of the stomach acts as a transitional region between the first and the last parts of the stomach. When the larvae first sickened, they vomited a green liquid and their faeces liquefied. Comma-shaped bacteria and Streptococci were numerous in both faeces and vomit. Bacilli and Staphylococci migrated upwards and downwards through the intestinal tract and begin their action on the larval bodies causing their liquefaction. Together with the bacteria of the middle and last part of larval stomachs characteristically shaped mycelia sterilia having cylindrical hyphae interrupted by swollen cells were observed.

The virus *Borellina litura*. The liquefied larvae were filtered through a series of filters with pores descending from 2 mm to 42 μ wide. The filtrate was centrifuged at 1500 revolutions per minute and the precipitate was found to contain fat bodies and tracheae. The precipitate was washed with distilled water and centrifuged twice, after which it was suspended in water and filtered through 3 G_3 Schott sintered-glass filter. The filtrate was centrifuged and the sediment dried under vacuum to a gray solid mass.

Polyhedra bodies were found to dissolve in a solution of 0.17 g sodium chloride + 0.293 g sodium carbonate per 100 ml distilled water. They were insoluble in hydrochloric acid, 96% ethanol and ether. The Polyhedra bodies were also soluble in a freshly prepared clear 1% soap solution.

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THE INTEGRITY OF THE CENTRIOLE IN *SACCHAROMYCES*[1]

by

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(Received November 11, 1958).

In E. B. WILSON's (1928) famous diagram of the resting diploid cell the central body is drawn as a centrosome containing two centrioles. The present communication reports that the haploid yeast cell contains a single centriole, the diploid two, the triploid three and the tetraploid four. This observation supports HARPER's (1905) view that the centriole is a single, stable, essential structure, the integrity of which is preserved throughout the life of the cell.

In the increasingly precise analysis of the structure of the yeast cell, the nature of the central body is beginning to take shape. A comparison of the two diagrams of the yeast cell by LINDEGREN (1951) and by LINDEGREN, WILLIAMS and MCCLARY (1956) show that the structure, now recognized as the "spindle", was previously called the "centrosome"; and that the dense structure previously called "centrochromatin" probably comprises the chromosomes retracted upon the spindle. There is no discussion in the 1951 paper of the centriole; the asymmetrical nature of the nucleus was ascribed to an attachment of the nuclear vacuole to the central apparatus corresponding to that found by HARPER in *Phyllactinia* and by DODGE (1927) in *Neurospora*. It has lately become clear, that the asymmetrical nature of the *Saccharomyces* nucleus is due to the polar orientation of the spindle and its constant association with the nuclear vacuole. The centriole was first observed and described by RENAUD (1938); he called it the "centrosome"; although his drawings and discussions make it clear that he is speaking of the

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"centriole" rather than the "centrosome". RENAUD discusses the nucleolus as a clearly defined body in the resting nucleus, but the nucleolus has the same stainability as the cytoplasm. The nucleolus, shown by LINDEGREN, WILLIAMS and McCLARY (1956, figures 25, 26 and 27) to occur in the nuclear vacuole, does not stain with nuclear stains and would not have been visible in his preparations. The same mistake concerning the stainability of the nucleolus is made by DELAMATER (1950) in discussing his Figure 7. DELAMATER also observed the centriole. RANGANATHAN and SUBRAMANIAM (1947), SUBRAMANIAM (1951) and SWAMINATHAN and GANESAN (1958) described the centriole and centrosome, and it is apparent in retrospect that the "cytoplasmic clear area" observed by LINDEGREN and RAFALKO (1950) in nearly every cell is also the centrosome. The centriole and the centrosome in the yeast cell were described by LINDEGREN, WILLIAMS and McCLARY (1956) and clearly photographed in Figure 5 of their paper.

RENAUD speaks of the nucleus as a spherical form enclosed in a membrane and containing an "achromatic nucleoplasm." The "achromatic nucleoplasm" is the structure which we designate the "spindle". He states that the chromatin is disposed as a fine network principally in the neighborhood of the nuclear membrane, – which we interpret to mean that the spindle is solid and that the chromatin is on the surface of the spindle. He described the "centrosome" as a "small grain" enclosed in a "clear area". This is incorrect; the small grain is the "centriole"; the clear area surrounding the grain is the "centrosome". RENAUD described the division of the centriole and the formation of a solid spindle, from the nucleus, with a centriole at either end. It is crystal clear from RENAUD's drawings (fig. 1) that the solid spindle in his Figures 5 and 6 must have originated from what he called the "achromatic nucleoplasm" or what we now call the "spindle".

MATERIALS AND METHODS.

The observations in this analysis were principally made upon living material by phase contrast microscopy. A Leitz phase condenser was used on the Leitz Ortholux microscope with a 40-× Zeiss phase contrast oil immersion objective. This system has the advantage of the flexibility of the Leitz condenser (which can pass from phase to dark field) and the long focal distance of the 40-×

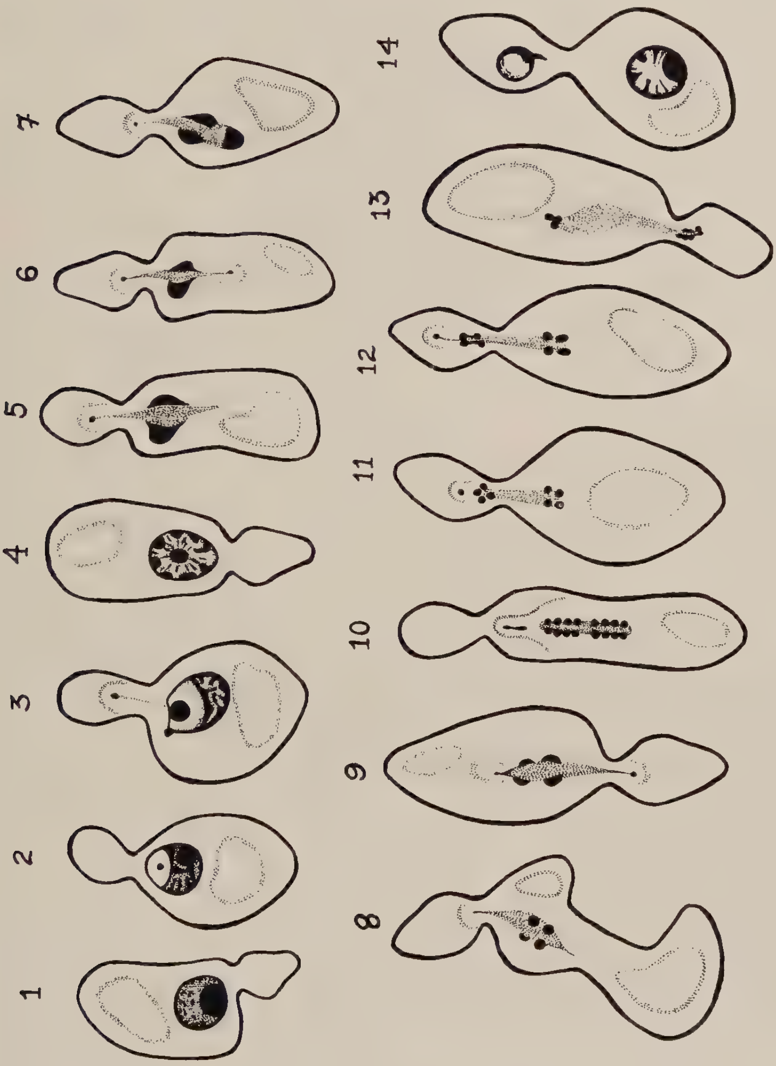

Fig. 1. A copy of RENAUD's drawings indicating the centrioles and the solid spindle.

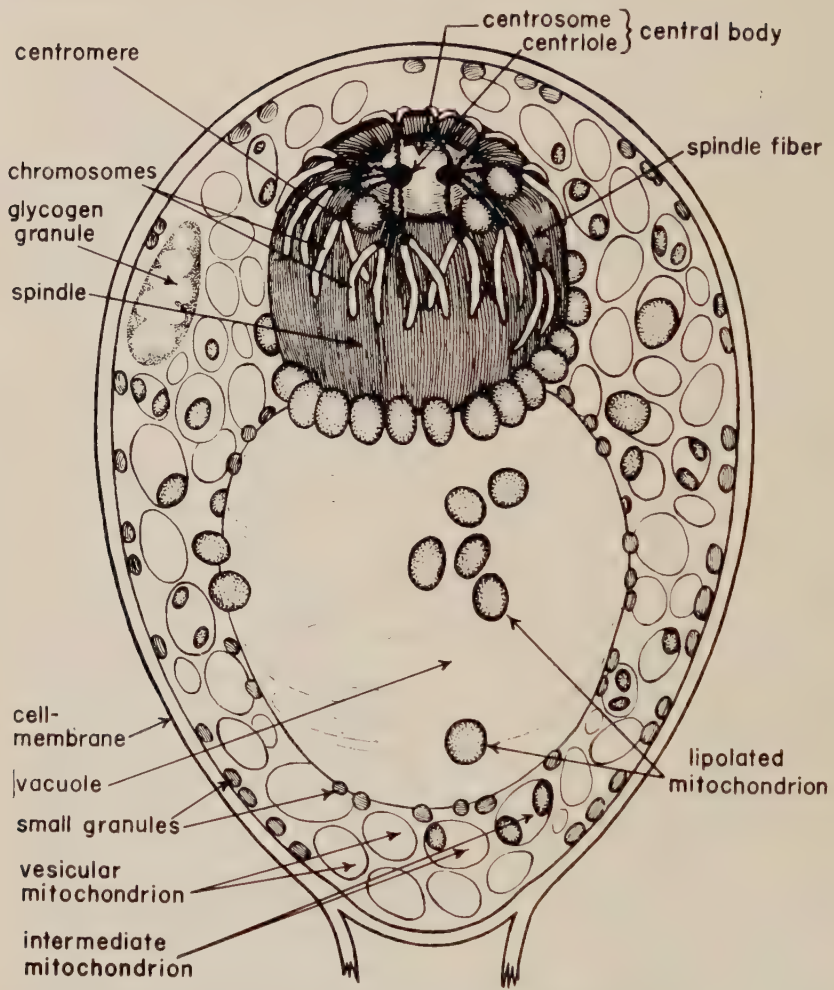

Fig. 2. A diagrammatic representation of the yeast cell according to the most recent views, indicating the solid spindle on which the centrosome containing two centrioles is located. The chromosomes are oriented to the centrioles by spindle fibers. A single haploid set is oriented toward each centriole. The cytoplasmic granules are labelled and the diagram is discussed in the text.

Zeiss objective which gives a flat field. An additional unexpected advantage was the fact that in many instances the centrosome is pink under these optical conditions and sharply distinguished from the other granules within the yeast cell. One or more dark centrioles are clearly defined within the pink centrosome using this optical system.

The diagram of the yeast cell in Figure 2 is a composite diagram and indicates structures such as the spindle fibers which are only rarely visible. It also shows the spindle which is seldom seen in vital preparations and then only indistinctly. In unstained preparations the vacuole, as well as the spindle, may be obscured, especially if the cell contains considerable amounts of glycogen; the figures, which are direct drawings show only those structures which were visible at the time of observation.

Lipolated and Vesicular Mitochondria.

By centrifugal separation Hebb, Montgomery and Slebodnik (1958) demonstrated two different kinds of granules in the cytoplasm of the yeast cell: the refractile granule and the vesicular granule. The refractile granules are enzymatically inactive while the vesicular granules are enzymatically active. Extended observations have led us to infer that the refractile granules and the vesicular granules represent extreme conditions of the same structure, the former being "lipolated" mitochondria and the latter the "vesicular" mitochondria (fig. 2). These bodies are transformable into each other by a process involving the accumulation of lipids or the dispersion of the lipid. On this view, the refractile granules are lipolated, inactive mitochondria while the vesicular granules are the active mitochondria. The accumulation of lipid leads to enzymatic inertness and the removal of lipid to enzymatic activity.

Cowdry's (1924) conclusions concerning the variations in the mitochondria tend to support the inference that lipolation leads to enzymatic inertness:

> "Although their causation remains a mystery, changes in the shape of mitochondria do constitute by far the most delicate criterion of many types of cell injury at our disposal. They tend to follow, moreover, in a definite sequence. First we often observe a breaking-up of filaments into granules (this may also be induced by faulty technique), then either a disappearance of the granules or their enlargement into coarse spherules. Coincident with this increase in size, the granules often blacken more readily with osmic acid, indicating perhaps an increase in their fatty acid content (see p. 325), which may be responsible for the calling into operation of the law of least surfaces.
>
> "It would appear that in most cases the filaments break up into segments which round up into spherules, but it is possible that

individual filaments may give rise to single droplets . . . Occasionally an increase in the girth of mitochondrial filaments is noted but very rarely are they observed to elongate as a result of injury. Quite frequently they swell up into droplets with pronounced fatty and lipoidal properties. These morphological changes will remain very obscure until we are able to correlate them with chemical changes by the aid of more exact methods for the detection of the lipoidal and protein fractions which probably enter into the composition of mitochondria. The assumption is perhaps warranted that a decrease in the number of mitochondria is a sign of depression of functional activity, provided that they retain their normal shape but, when the increase is manifested by a swelling up of mitochondria into rounded droplets of different sizes, the condition is apt to pass insensibly into a simple accumulation of fat and lipoid which would point, on the other hand, to a decrease in the rate of intracellular oxidation."

Changes in the mitochondria and the cellular reserves produced by nutrition and the effects of these changes on the enzymatic activity of the cells have been described by Lindegren (1945):

"The vegetative budding yeast cell, in the logarithmic growth phase contains a very large, centrally located vacuole and stains a light golden yellow with iodine (fig. 1). It contains a few tiny fat globules. If these cells are placed under conditions of relatively low oxygen tension and supplied with an abundance of sugar, after budding has ceased, granular deposits of glycogen appear in the cytoplasm, which finally deform the vacuole and diminish its size (figs. 3, 4, 5 and 6). If the cells are well-aerated and supplied with an abundance of sugar, fat globules appear and increase in size and number and also tend finally to obscure the vacuole, although it retains its spherical form (fig. 7); non-granular carbohydrate also accumulates in the cytoplasm.

"Unstained cells containing glycogen can be recognized by the high refractive index of the cytoplasm (fig. 3). The nuclear vacuole in a glycogen-containing cell is often concealed by the glycogen. The visible vacuoles often appear to be multiple (figs. 4 and 5), but critical observation shows that the small vacuoles are all interconnected with each other by fine canals and are merely separate compartments of one major vacuole. This is consistent with the view already expressed (Lindegren, 1945) that each yeast cell contains only a single vacuole. In many of the glycogen-containing unstained cells in which no vacuole is visible, staining with Lugol's solution often reveals the vacuole compressed into the middle of the cell by a surrounding sheath of glycogen. The vacuole in a glycogen-containing cell is usually very small.

"A medium favorable for sporulation was developed in this laboratory (Lindegren and Lindegren, 1944) containing extracts from beet, apricot, grape and yeast. After a culture has grown for several weeks on slants of this rich natural medium, the cells contain abundant deposits of fat and, although all the cells are alive, they are dormant.

"A diploid culture (155 × Em1A) was grown 10 days on an agar slant of pre-sporulation medium. Practically all the cells were full of fat (fig. 11). The vacuoles were clearly outlined in the cells loaded with fat, indicating that fat and glycogen deposition occurs without deformation of the vacuole and that the cytoplasm becomes relatively rigid. The cells were transferred to 6% glucose nutrient broth on the shaker and examined 1 hour later. Much of the fat had disappeared from half of the cells (fig. 12). Iodine-potassium iodide showed a deposit of non-granular carbohydrate. At the second hour, most of the cells were budded and half of the budded cells showed a few fat granules. The vacuoles appeared to be multiple and obscured in the growing cells. In three hours, there was very little visible fat left in the cells, nearly all of which contained multiple or obscured vacuoles. Practically all the cells had budded, showing that the culture was viable.

"A Warburg analysis was made on the cells taken directly from the pre-sporulation slant and shaken with phosphate buffer containing 4% dextrose, but with no other nutrients. The Q values after 150 minutes were Q_{O_2} – 25; $Q^{O}_{CO_2}$, – 41, and $Q^{N}_{CO_2}$, 0. During the same period, all the cells in the Erlenmeyer containing nutrient broth had budded. Only 2% of the cells from the Warburg vessel had budded; 98% of the cells showed little or no change. Although these cells were unable to ferment anaerobically, they were not completely dormant, as was indicated by their absorption of oxygen.

"This experiment showed that dormant cells were almost completely inactive in a phosphate buffer and dextrose, regained their respiratory activity in a few hours in a nutrient medium."

The recovery of respiratory activity was probably achieved by the transformation of lipolated mitochondria to the vesicular type of mitochondria.

One, two or three refractile granules are almost invariably associated with the centrosome and pass with it into the bud at each cell division or remain associated with it during karyogamy and meiosis, thus insuring the transfer of at least one into each new cell or spore. As the cytoplasm flows into the bud several of the smaller vesicular mitochondria also enter the bud, thus insuring a transfer of both the active and inactive mitochondria into the bud. Under certain conditions many or most of the granules aggregate about the centrosome and the aggregation of ultraviolet-absorbing granules observed by CASPERSSON and BRANDT (fig. 8a) is probably centered on the centrosome. The refractile granules have been observed to bud and BAUTZ and MARQUART (1953) commented specifically on their regular distribution from mother cell to bud, although they did not observe their association with the centrosome.

Small Granules.

The cytoplasm of actively growing cells contains many small granules closely applied to the outer surface of the vacuole and the inner surface of the plasma membrane. They appear dark in dark field and phase contrast microscopy but are not visible by light microscopy. This differentiates them from the refractile granules which are visible by light microscopy and are light in dark field. The small granules pass into the bud at its first formation and may synthesize materials essential to the repair of the cell wall and the nuclear membrane, since they also line up on the planes in the ascus which eventually form the spore walls. Their behavior is reminiscent of the behavior of the rib-forming bodies described by LINDEGREN and SCOTT (1937).

Photography of the Cytoplasmic Granules.

The cytoplasmic granules have been photographed by Dr MARION O. WILLIAMS with phase microscopy by crushing yeast cells between the slide and cover slip and photographing the extruded contents. In Figure 3 squashed preparations are shown in which the vesicular mitochondria (V.M.) are clearly visible. The lipolated mitochondria (L.M.) and the small granules (S.G.) are also easily distinguishable. A few intermediate mitochondria (I.M.) are visible and centrioles (C.) which have not been pressed out can be seen in three of the "ghosts".

Karyogamy.

Karyogamy in the ascomycetes is conventionally preceded by the conjugate division of two associated nuclei and follows a pattern first described by DANGEARD (1894) which we may designate as the "crozier"-type of karyogamy, standard descriptions of which are presented in all textbooks of mycology.The "crozier"-type of karyogamy also occurs in *Saccharomyces* and was described by LINDEGREN and LINDEGREN (1954). Previously LINDEGREN (1951) had described karyogamy by a process which may be described as a

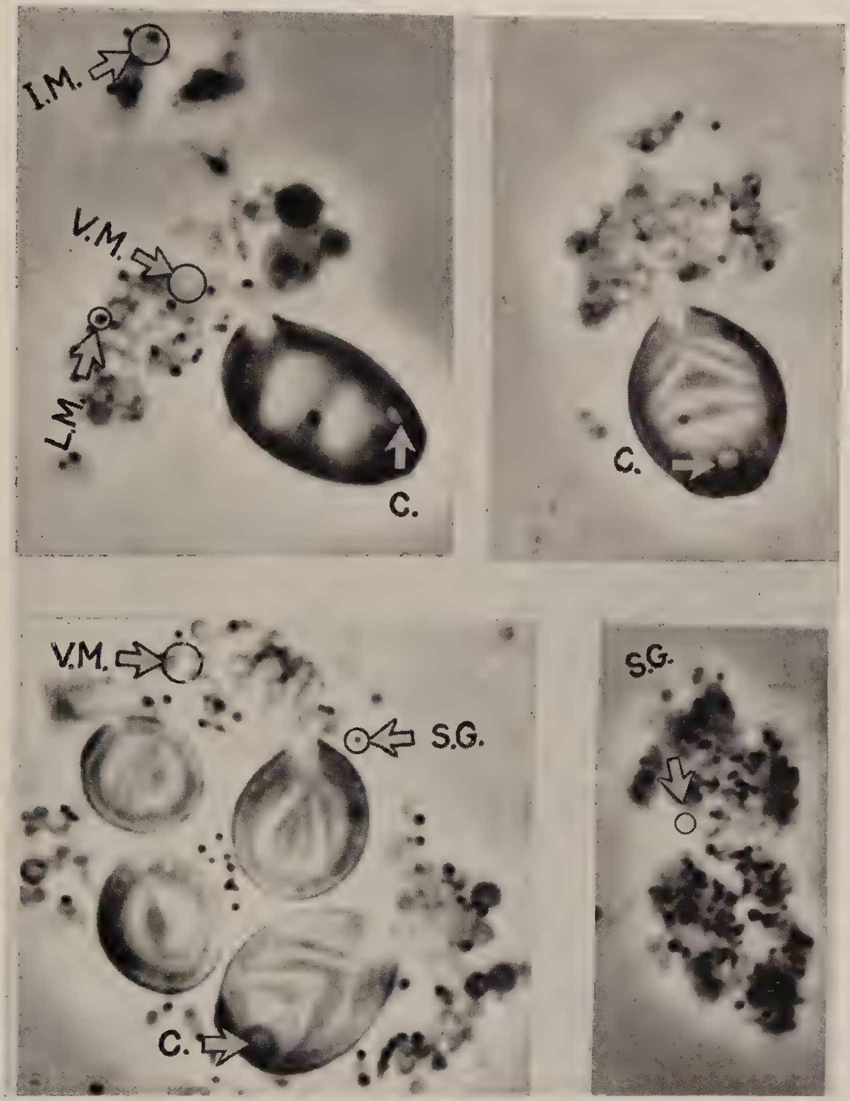

Fig. 3. Photographs by Dr Marion O. Williams of yeast cells crushed between cover glass and slide showing the characteristic granules: centrosome, C.; vesicular mitochondria, V. M.; lipolated mitochondria, L.M.; intermediate mitochondria, I.M.; small granules, S.G.

"complete" karyogamy, since both gametic nuclei fused to form the zygote without a concurrent conjugate division. Both the "complete" and the "crozier"-type of karyogamy were observed in the present study.

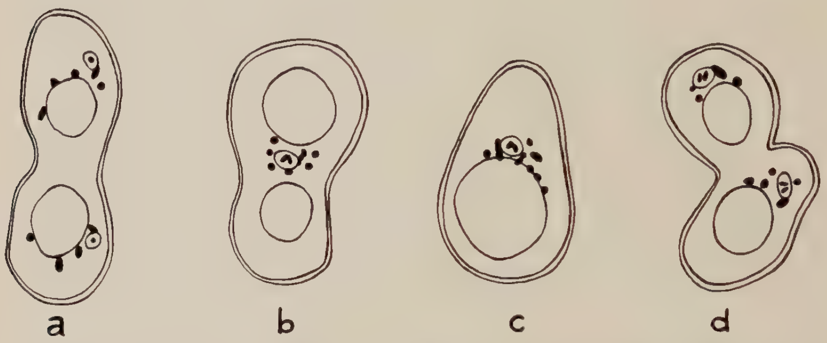

Fig.4. A "complete" karyogamy between two diploid gametes resulting in the formation of a zygote centrosome containing two centrioles. The structures indicated in the drawing are the vacuole, the centrosomes with centrioles and the lipolated mitochondria.

Behavior of the Centriole in Karyogamy.

Figure 4a shows a zygote (produced by the fusion of two haploid cells) which contains two vacuoles and two centrosomes each containing a single centriole. Spindles are not visible in preparations of this type and although not indicated in the figure may be assumed to be located between the centrosome and the vacuole. A single refractile granule is closely associated with each centrosome. In Figure 4b the centrosomes have fused but the vacuoles have not yet fused. It may be inferred that the spindles are also fused in Figure 4b. In Figure 4c fusion is complete since only a single vacuole is visible. Figure 4d shows the zygote producing a bud containing a diploid nucleus, inferred from the fact that the centrosome contains two centrioles.

Figure 5 shows a "crozier"-type copulation. In Figure 5a the characteristic association of refractile granule and centrosome is apparent and each centrosome contains a single centriole. In Figure 5b the two haploid nuclei are still present in the zygote although the vacuole is obscured in one instance by overlying material. The bud contains two centrosomes each with a single centriole together

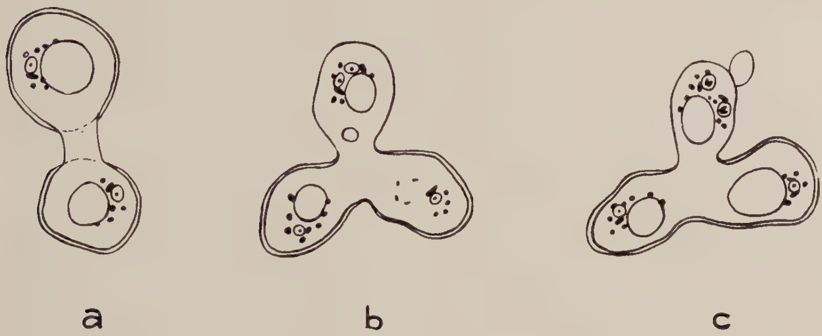

Fig. 5. A "crozier" type karyogamy between two haploid gametes producing a diploid zygote. Discussed in the text.

with their associated granules and two vacuoles. The small vacuole in the zygote may represent a lobe connected with the zygote vacuole. LINDEGREN (1945) pointed out that vacuoles which appear to be lobed are invariably interconnected but that specific conditions are required to demonstrate the interconnections:

> "The first step (in budding) is the formation of a long slender tube leading from the vacuole to the periphery of the cell. This phenomenon can only be observed in cells containing enough

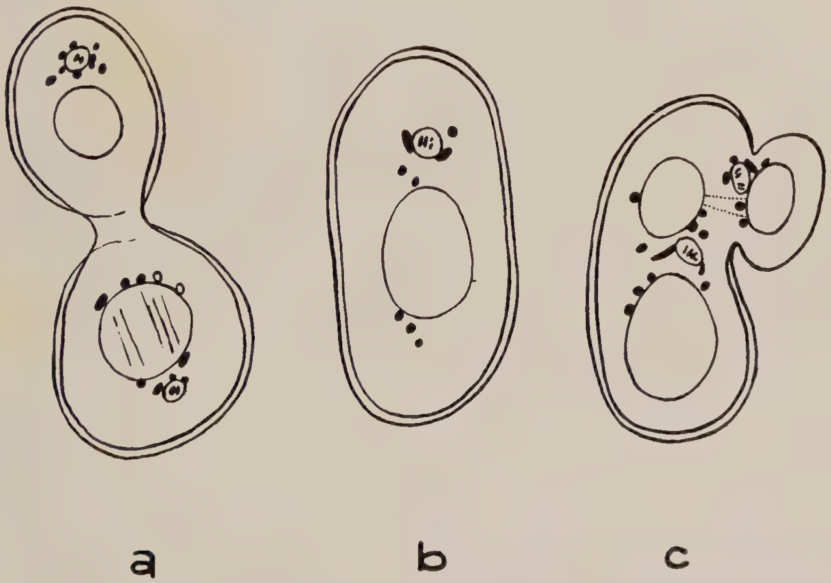

Fig. 6. A "complete" karyogamy between two diploid gametes producing a tetraploid zygote with four centrioles in the centrosome.

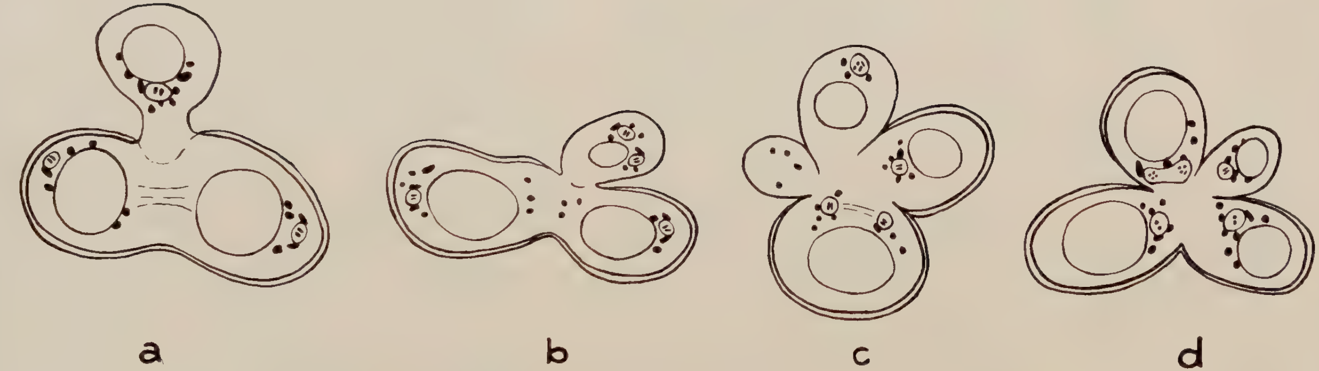

Fig. 7. A "crozier" type karyogamy between two diploid gametes.

glycogen so that the iodine stain delimits the vacuole and its tube as a clear space in the surrounding reddish brown cytoplasm. Observation is facilitated by the use of a Wratten 45 filter which converts the reddish brown color of the cytoplasm to blue-black and reduces the chromatic aberration of the lens system. The canal from the vacuole may begin any place on the surface of the vacuole . . ."

In Figure 5c the zygote of a "crozier"-type karyogamy has produced a bud with two centrosomes each containing two centrioles. One diploid centrosome of the zygote nucleus is preparing to migrate into the first bud from the zygote nucleus.

Figure 6 shows the copulation of two diploid gametes (LINDEGREN and LINDEGREN, 1951). The formation of a "complete" zygote is shown in Figure 6b; the zygote centrosome contains four centrioles. Figure 6c shows the division of the complete zygote with part of the vacuole passed into the bud. A connection between the two vacuoles of the zygote may be inferred.

Figure 7 is an example of the "crozier"-type karyogamy between two diploid gametes. In Figure 7a one gametic nucleus with its diploid centrosome has passed into the bud but the second diploid gametic nucleus has not yet divided. In Figure 7b the bud contains two diploid centrosomes which have not yet fused and hence both gametes have made their contributions to the zygote. In Figure 7c the zygote nucleus is completed containing a tetraploid centrosome and one of the gametic nuclei has initiated a second division by the division of its diploid centrosome. In Figure 7d the tetraploid zygote nucleus has initiated division as indicated by the telophase of the tetraploid centrosome and one gametic nucleus has completed its second division although the second gametic nucleus has not yet divided.

Figure 8 is a detailed drawing of specific stages in the division of haploid and tetraploid centrosomes. The clear pink structure of the centrosome and its relatively large size has made it possible to observe figures of this type with the present optical system.

Summary.

The observation that the number of centrioles in the centrosome of a yeast cell correspond to the degree of ploidy supports HARPER's view that the centriole is a constant, stable, essential cell structure. The implications of this view on the function of the centriole and

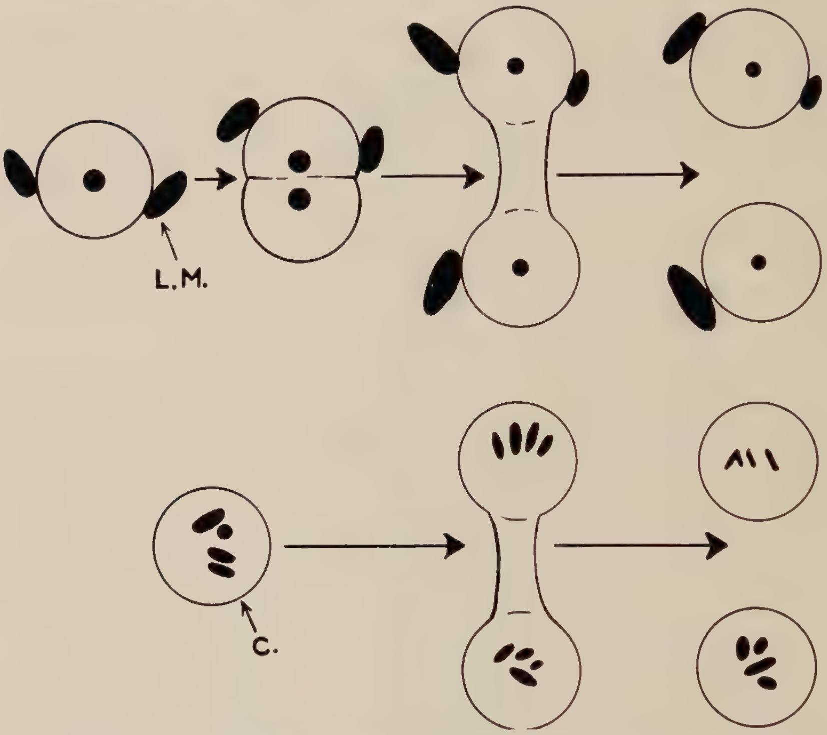

Fig. 8. Division of haploid and tetraploid centrosomes according to direct observations.

the integrity of the spindle fiber will be considered in subsequent communications.

Besides the centriole, four other types of granules appearing in the yeast cell are described and distinguished from the central body. These are the lipolated mitochondria, the vesicular mitochondria, the intermediate form of mitochondrion and the small granules.

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AN ELECTRON MICROSCOPE STUDY OF L FORMS OF GROUP A STREPTOCOCCI

by

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(Received September 27, 1958).

The morphology of the L forms of bacteria has been the subject of many studies, which have been reviewed among others by DIENES and WEINBERGER (1951) and by KLIENEBERGER-NOBEL (1956). The electron microscope has been used relatively seldom, possibly because of technical problems. The obvious method of choice is ultrathin sectioning. A prerequisite for this technique is the capacity of the object to withstand vigorous dehydration during the embedding process. Because of the high fragility of these organisms artifacts are easily introduced.

In the present study we made use of the property of the L forms of streptococci to grow into the medium (fig. 1) – a feature, common to L forms in general – instead of the surface growth of the bacterial form: the L forms have embedded themselves as it were in the medium. Intact L colonies were therefore fixed together with the surrounding medium in osmium tetroxide, followed by the routine methacrylate procedure.

Although so far only two strains of streptococcal L forms have been studied with this technique, it seems likely that the procedure may have a wide applicability for the investigation of L forms in general and related phenomena.

MATERIAL AND METHODS.

The GL8 and AED strains of the group A, beta hemolytic streptococci, used in this study, were the same as described previ-

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[1]) Fellow of the Stichting "Rheumatiekfonds Dr JAN VAN BREEMEN".

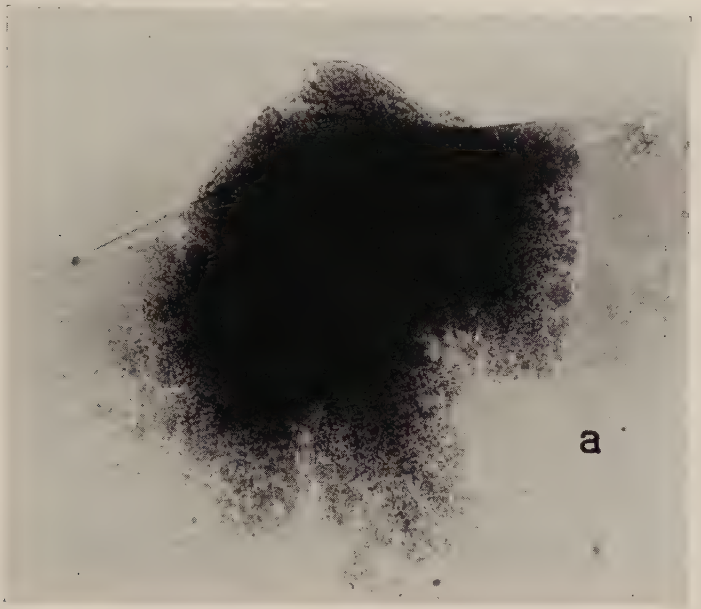

Fig. 1. Micrograph, showing the ingrowth of a streptococcal L colony into the agar medium (a); 100 ×.

ously (Sharp, Hijmans and Dienes, 1957). Transformation from the streptococci to L forms was induced by penicillin on solid media containing horse serum and approximately 3.5 per cent NaCl, according to Sharp (1954). The colonies from which sections were made, were 2–3 days old and were taken from cultures, which had been transferred in the L form at least 20 times.

In order to obtain comparable controls, sections were also made from colonies of the coccal form cultured in pour plates, where surface growth was absent.

Agar cubes of about 1 cmm were excised, each cube containing one or more colonies. These cubes were fixed in a 1% OsO_4 solution in buffered physiological saline with a pH of 7.2 (Sjöstrand, 1956) for one hour, washed in several changes of saline, dehydrated in an alcohol series and embedded in methacrylate. As this osmium solution is hypotonic with regard to the culture medium, controls were made with an identical solution, but with a final concentration of 3% NaCl.

For ultrathin sectioning a pyramid was cut as illustrated in figure 2. The sections were made with a modified Philips microtome and studied in a Philips EM-75 electron microscope. Some preparations were also studied in a Siemens Elmiskop.

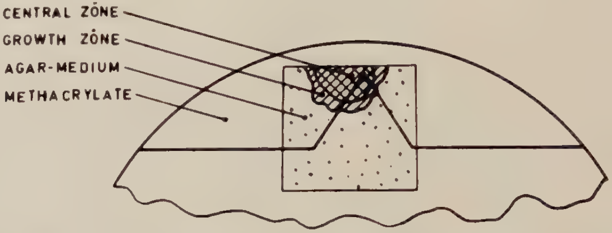

Fig. 2. Diagram illustrating the method of preparation of L colonies.

Results.

The electron micrograph (fig. 3) of a section through normal beta-hemolytic streptococci group A (strain GL8) shows that very few internal details can be seen due to the strong osmiophilia; only in few bacteria an indication of a cell wall is visible.

No significant differences are found between the L forms of the streptococcal strains GL8 and AED. Neither can any marked difference be observed between the pictures of L forms of streptococci fixed in the routine "isotonic" fixing fluid and those of the organisms fixed in a fluid supposed to be isotonic for the L forms.

In each colony a central dense zone and a less compact outer zone is seen. The central zone consists of closely-packed vesicles each with a clear outer limiting membrane (fig. 4). With high magnification the limiting membranes appear to be double (fig. 5–2) in some places. The area just inside the membrane shows an amorphous appearance or a finely reticulated pattern (fig. 6). In between the vesicles there is nondescript material, for the greater part undoubtedly consisting of tangentially cut vesicles; many vaguely-defined membranes are seen (fig. 5–3) in some preparations. In the vesicle walls, bulging into the inside, granules are visible, varying in size from 300 Å to 3000 Å; these granules show moderate osmiophilia (fig. 4–3).

Electron-optically the vesicles appear empty except for the constant presence of a system of thin membranes (fig. 4 and 5). In some places these membranes seem to be attached to the vesicle walls; elsewhere they appear to be floating freely. To the majority of these membranes granules of marked osmiophilia about 500Å in diameter are attached (fig. 4–5).

The growth-zone partly consists of clusters of closely-packed thin-walled vesicles showing very few details. The other parts of the growth zone are occupied by vesicles which appear very irregular both in shape and size. The same details as described in the central vesicles are to be observed here: clear outer limiting membranes, granules of varying sizes embedded in the vesicle-walls and intravesicular membranes with attached granules (fig. 7, 8 and 9).

The granules, constituting components of the vesicle walls are usually bulging into the interior of the vesicles, only the largest ones, about 3000 Å in size, are bulging into the medium. Occasionally they may seem to be lying freely. Around the largest granules a limiting membrane may be observed (fig. 9 and 7–3); in addition there often is a beginning of central vacuolization, suggesting a transition into a vesicle (fig. 9–3 and 9–4).

In the walls of peripheral vesicles tiny "microvesicles" of 100–300 Å may be observed; especially in tangential sections they appear very clearly (fig. 8–5).

DISCUSSION.

From the study of ultrathin sections of L forms of group A beta-hemolytic streptococci the fundamental structures appear to be vesicles of varying sizes and shapes with – embedded in their linings – granules of variable diameter. The simplest way to interpret the various pictures seems to be the assumption that granules "ripen" in the vesicle wall, then bulge outward and become surrounded by a membrane; finally they develop into vesicles in their turn.

The characteristic pleiomorphism discerned with the light microscope, is also very prominent on the submicroscopical level. In addition the electron micrographs reveal features as a double lining with microvesicles and intravesicular membrane-like structures to which are attached osmiophilic granules.

It seems of considerable interest that in our material no free-lying granules were observed with certainty: they all were attached to or embedded in the vesicle walls. This could have a bearing on the problem of filtrability. Although the smallest units should easily pass bacterial filters, all our attempts to culture L forms from the filtrates have been consistently negative.

It is difficult to see how the wealth of details shown by the streptococcal L form can be explained as a consequence of a lack

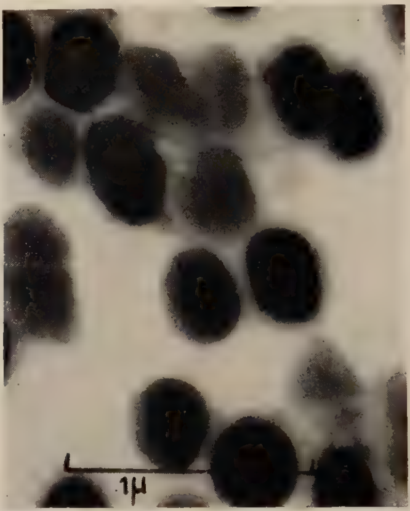

Fig. 3. Electron micrograph of sectioned streptococci in the coccal phase. No clear structural details can be discerned apart from an occasional cell wall; 32.000 ×.

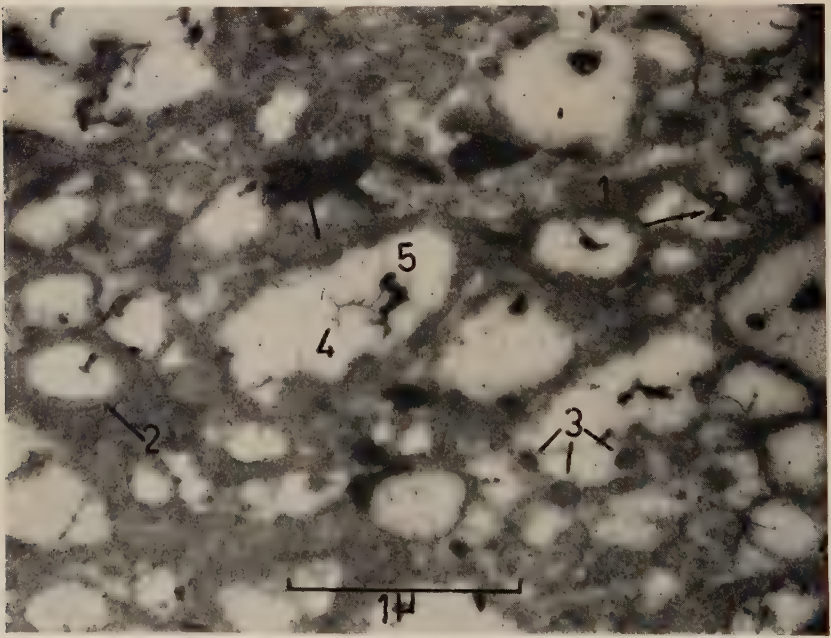

Fig. 4. Electron micrograph of a section through the center of a colony of streptococcal L forms; 32.000 ×.

1. Full-size granule.
2. Limiting membranes of vesicles.
3. Medium size granules in the wall of an irregularly shaped vesicle.
4. Intravesicular membranes.
5. Osmiophilic granules attached to 4.

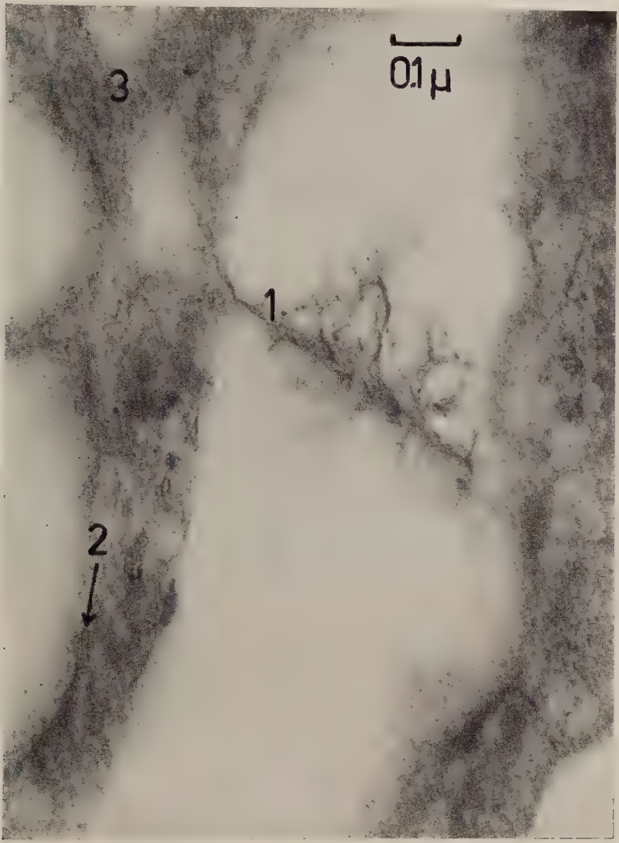

Fig. 5. As fig. 4; 80.000 ×.
1. Intravesicular system of membranes.
2. Double limiting membrane.
3. Extravesicular membranous structures.

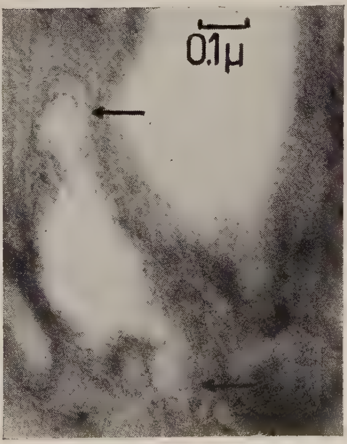

Fig. 6. As fig. 4; 60.000 ×.
The arrows point at reticulated patterns in the vesicle walls.

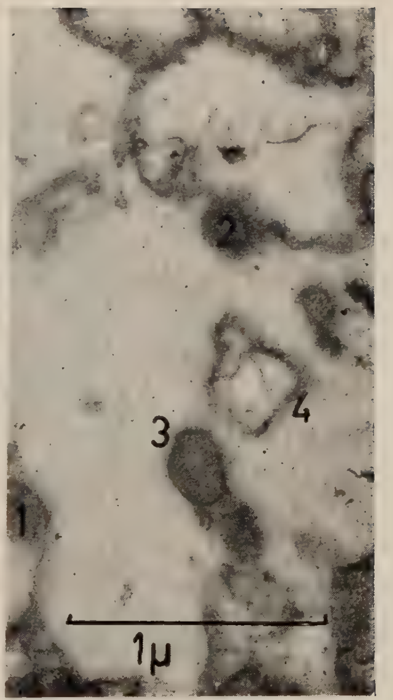

Fig. 7. Electron micrograph of sectioned streptococci in the L form in the growth zone of an agar culture; 32.000 ×.

1. Medium-size granule in vesicle wall.
2. Granule of larger size, bulging outward.
3. Homogeneous granule with distinct outer limiting membrane.
4. Small vesicle with intravesicular membranes, attached to the vesicle wall.

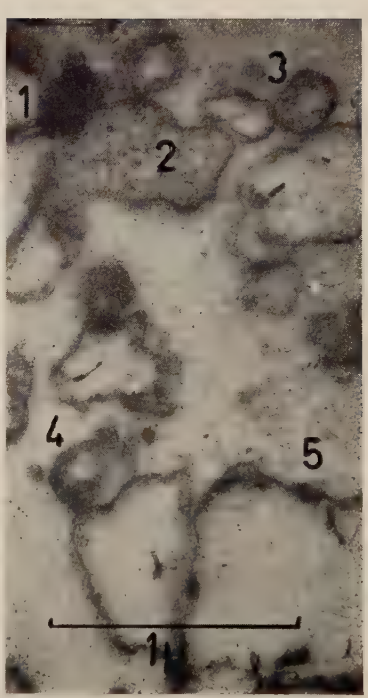

Fig. 9. As fig. 6; 32,000 ×.

1. Granule in the last phase of development.
2. Tangential section through vesicle wall.

3 and 4. Granules showing beginning of vacuolization.

5. Developing granules.

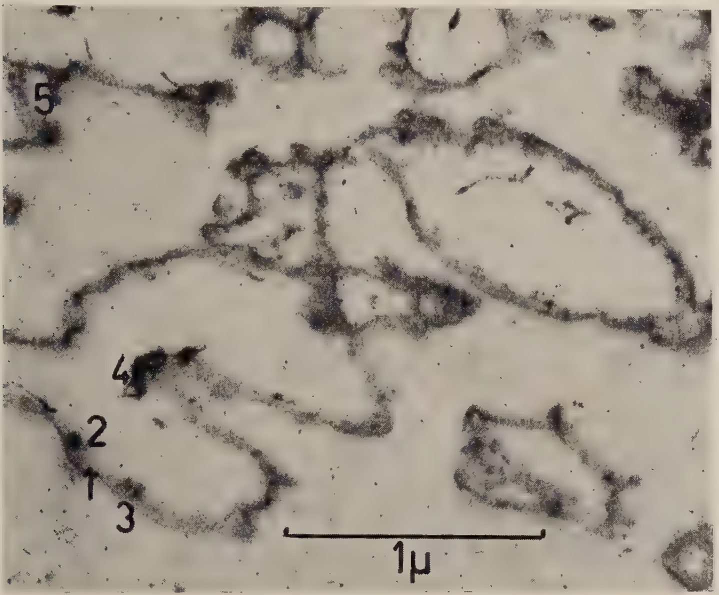

Fig. 8. As fig. 7; 32.000 ×.
1. Limiting membrane of irregularly shaped vesicle.
2. and 3. Small sized granules.
4. Intravesicular osmiophilic granules.
5. Microvesicles in connection with tangentially cut vesicle wall.

of rigidity, caused by the absence of the bacterial wall. It may be possible that all these structures are normal cell components, rendered visible by the desintegrating effect of swelling.

The only material for comparison is offered by THORSSON and WEIBULL (1958) in their recent publications on sectioned specimens of a stable *Proteus* L form. They too find a complex organization, but also report on vesicles, containing a large number of free-lying small bodies of rather uniform size. It is not clear to what extent differences in the species under investigation or in preparatory techniques play a role in this discrepancy.

Summary.

A technique is described for the preparation of ultrathin sections of bacterial L forms grown in a solid medium. Fundamental structures found in colonies of L forms of beta-hemolytic streptococci group A consist of vesicles up to several microns in size; embedded

in the vesicle walls are granules 300–3000 Å in size. Each vesicle is surrounded by a limiting membrane, which in some places appears to be double. The vesicle walls contain tiny vesicles less than 300 Å in diameter. Inside each vesicle a system of membranes with osmiophilic granules can be observed.

The technique described seems to have a general applicability for the study of the ultrastructure of the L forms or related phenomena.

Aknowledgements.

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THE OCCURRENCE OF MULTINUCLEATE GIANT CELLS IN YEASTS

by

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(Received October 13, 1958).

During the course of a detailed study on the cultural behaviour and cytology of *Lipomyces lipofer* it was observed that single spore cultures growing vegetatively on solid medium suddenly gave rise to giant cells in considerable numbers. Some months prior to this observation an essentially similar phenomenon had been noted in three different *Saccharomyces* genotypes maintained in liquid medium under otherwise similar conditions. Despite the absence of a satisfactory causal explanation for the phenomenon, the present interest shown in polyploidy and other genetic abnormalities in yeasts is considered sufficient justification for describing our observations in some detail.

Observations on *Lipomyces lipofer*.

Vigorous vegetative growth of *L. lipofer* is obtained on potato agar; the cells are round to oval, less than 10μ in length, contain one or more large fat globules, and are uninucleate (Roberts, unpublished). This species does not, however, form spores on potato agar but requires other solid media, such as yeast water agar, for the purpose. In a series of experiments designed to elucidate the developmental cycle of this organism, single spore cultures were transferred to yeast water agar and stained on successive days according to a modification of Ganesan and Swaminathan's technique (1958) for Giemsa staining of yeast nuclei[1]). As a control, stained preparations were made of the vegetative growth of the same culture on potato agar, and it was here that giant cells in *Lipomyces* were first observed. Two 2-day old single spore cultures, each from a different ascus, had been stained as controls, and both preparations showed, in addition to the normal vegetative cells, a considerable number of very large cells of varying size and shape, some of which had attained a length of 20μ. That their occurrence was not due to staining artifacts was ascertained by the observation that water mounts of living cells of the same cultures likewise contained giant cells in large numbers. The stained preparations showed very clearly the presence of one nucleus in each of the normal cells and showed, in an equally convincing manner, that the giant cells were characteristically multinucleate, with the number of nuclei per cell ranging from 1 to 6. Figures 1–4 illustrate the comparative sizes of the multinucleate giant cells and the uninucleate normal cells from one of these first preparations. The giant cell in Fig. 1 measured ca. 20 × 11μ.

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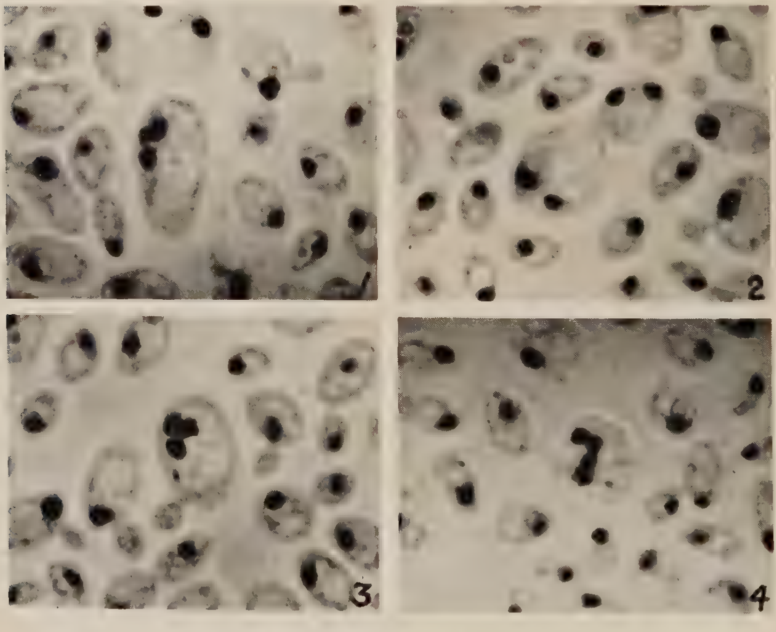
[1]) Because of the large amount of lipids present in the cells of *Lipomyces*, as well as the presence of a capsule of slime, Carnoy fluid was found to be a more suitable fixative for this yeast than the osmium-chloroform fixation employed by Ganesan and Swaminathan for *Saccharomyces* and *Schizosaccharomyces*.



Figs. 1–4. *L. lipofer.* Giant multinucleate and normal uninucleate cells from a single spore culture. (× 1000).

Six days later one of the two cultures on potato agar was re-examined microscopically and giant cells were still found to be in abundance. Employing the micromanipulation technique developed at the Carlsberg Laboratorium (WINGE 1935), isolations of single giant cells and single normal cells were then undertaken. Although *Lipomyces* thrives best on a solid medium with free access to air, previous experience in spore isolations had shown that they could be successfully accomplished in liquid droplets (ROBERTS 1957) and accordingly, droplets of dextrose yeast water were employed. Six giant cells of varying sizes were isolated and of these, three germinated but only two, C and D, developed into colonies sufficiently large to transfer out of the isolation chamber. Cell C differed from Cell D in being more elongate in form and nearly twice as large. Two round normal cells, A and B, were isolated at the same time, and both of these controls germinated and grew out into colonies. Fig. 5 represents an approximation of the comparative sizes of Cells A, B, C, and D, taken from rough sketches made at the time of isolation.

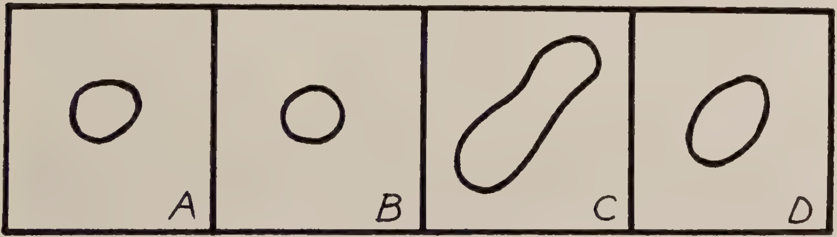
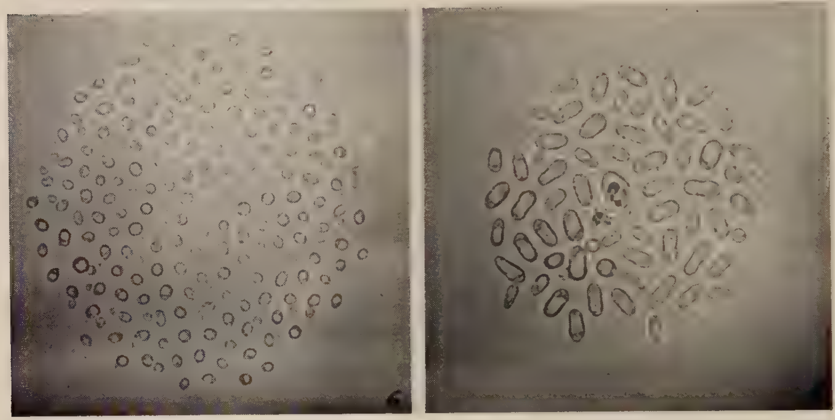
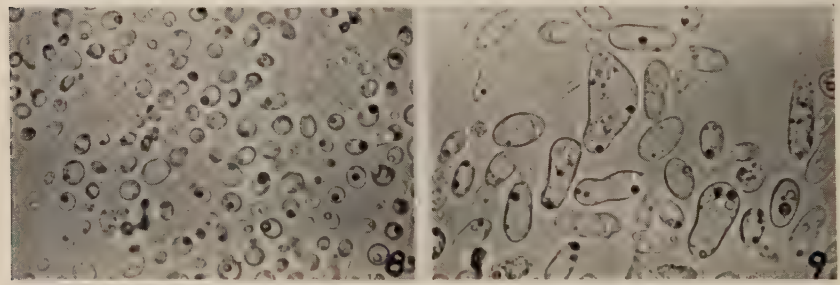


Fig. 5. A diagram of the comparative sizes of the four cells of *L. lipofer* which were isolated with the micromanipulator.

In the droplets, Cell C gave rise to a colony composed entirely of giant cells, while the colonies derived from Cells A and B were composed entirely of normal cells. This difference in appearance was very striking, as can be seen in Figs. 6 and 7, which were photographed four days after the cells were isolated and just prior to the transfer of the colonies out of the chamber to potato agar. At the time of transfer, Colony D consisted of cells which were only slightly larger than those of the controls, indicating that Cell D had possibly been a normal cell of greater size than the average, and it will be seen later that this assumption was probably justified. After 13 days incubation on potato agar at room temperature, the cellular morphology of Culture C still deviated strikingly from that of Cultures A and B (Figs. 8 and 9), while Culture D showed only a slight deviation. It should be noted, however, that at this time not all of the cells of Culture C were giants, a considerable number resembling normal cells in size and shape.

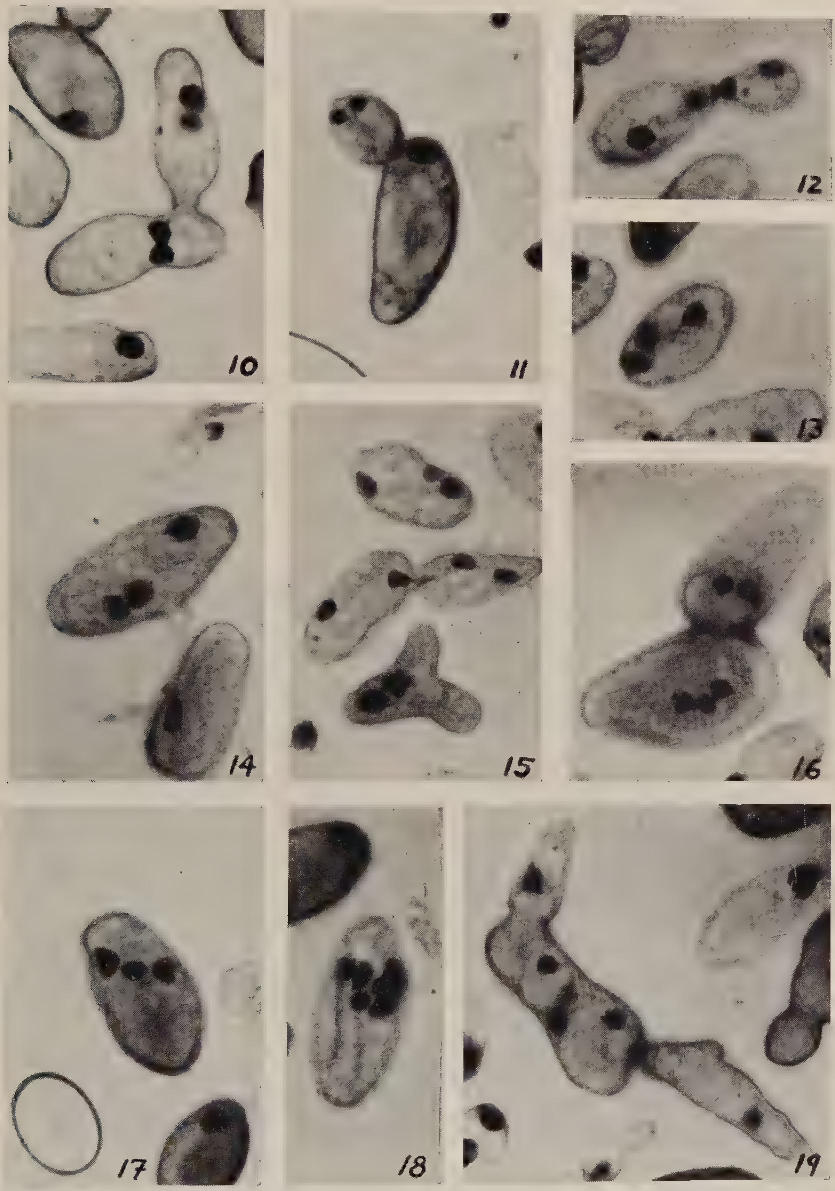


Figs. 6–7. *L. lipofer*. The colony which arose from Cell A (Fig. 6) and from Cell C (Fig. 7) photographed in the chamber four days after isolation of the cells. (× 190).



Figs. 8–9. *L. lipofer*. Water mounts of 13 day old cultures of A (Fig. 8) and C (Fig. 9) growing on potato agar. (× 310).

Twenty days after the cell isolations, stained preparations of 3 day old cultures of A, C, and D on potato agar were made. The cells of Culture A were small, round to oval, and nearly all uninucleate; a few binucleate cells were observed, but these were of normal size and may have represented ordinary mitosis preceding bud formation. The cells of Culture D were likewise uninucleate with few exceptions but were slightly larger than those of A. The cells of Culture C, on the other hand, included many giants of elongated or irregular shape containing anywhere from 1 to 6



Figs. 10–19. *L. lipofer*. Giant multinucleate cells of a 3 day old C culture on potato agar. (× ca. 940).

nuclei (Figs. 10–19). In addition, smaller uninucleate cells of normal appearance were present.

All four cultures (A, B, C, and D) were transferred to yeast water agar in order to determine whether any difference existed in their ability to sporulate. In the course of one week abundant sporulation had occurred in Cultures A, B, and D, and the surface of each colony had the brown color typical of sporulating *Lipomyces* cultures. In contrast, the growth of Culture C was of a pale cream color, and no spores were found. However, 30 days after inoculation, the Shimwell staining technique revealed the presence of a very few free spores in Culture C, together with some not yet liberated.

Giant colonies of Cultures B and D which had been growing for one month on wort gelatine at 15°C. were smooth and glistening and nearly identical in appearance, indicating that Cell D was actually a normal cell. Although the giant colony of Culture C was also devoid of sculpturing, it was quite clearly not identical with the other two (Fig. 20).

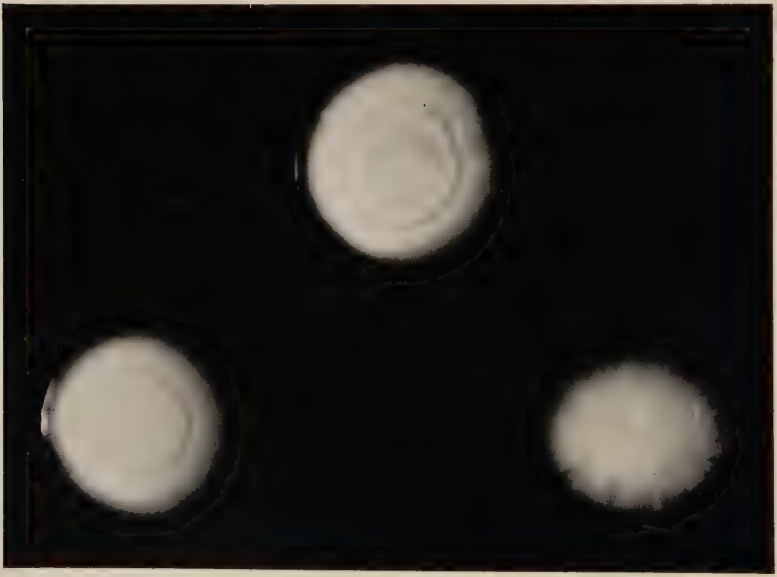


Fig. 20. *L. lipofer.* Giant colonies of Cultures B (upper), D (lower left), and C (lower right) on wort gelatine. (× 1.6).

Observations on *Saccharomyces.*

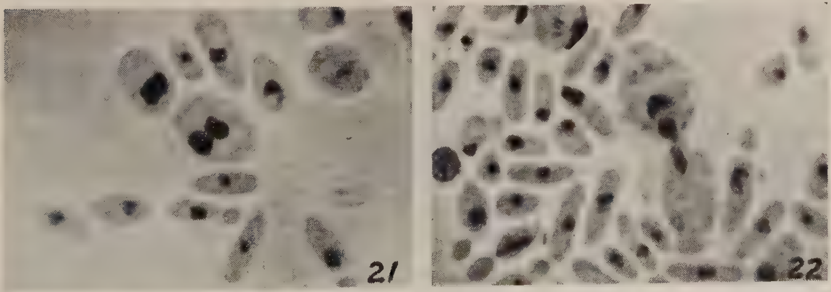
The cultures of *Saccharomyces* in which giant cells were found were the following:

H 307. – A hybrid, which had been artificially produced during an investigation of the genetics of melibiose and raffinose fermentation (Winge and Roberts 1957) and which descended from crosses involving *S. chevalieri*, *S. italicus*, and *S. carlsbergensis*.

62-141. – A diploid single spore culture from Hybrid 62 which resulted from a cross between *S. chevalieri* and the Yeast Foam strain of *S. cerevisiae* (Winge and Roberts 1949).

62–143. – A haploid single spore culture derived from the same ascus as 62–141.

Just as in the *Lipomyces* material, giant cells in H 307 were first noticed in stained preparations. In connection with some investigations on sporulation in *Saccharomyces* (Ganesan, Holter, and Roberts 1958), H 307 had been placed in contact with Fowell's sporulation medium (Fowell 1952) for 14 hours and then stained in the usual way; these preparations showed a considerable number of multinucleate giant cells among the normal uninucleate diploid cells. A characteristic feature of the giant cells of H 307 was that they mostly occurred in chains, a growth form not seen in the *Lipomyces* giant cultures. Two day old cultures of H 307 in Pilsner wort were examined and found to contain many giant cells, and at the same time their presence was observed in wort cultures of the diploid 62–141, where they occurred singly and in chains, and in wort cultures of the haploid 62–143, where they only occurred singly. Both of these cultures were stained, and again the giant cells were found to contain two or more nuclei in striking contrast to the smaller uninucleate cells. Figs. 21 and 22 show stained giant and normal cells of 62–143. The length of the binucleate giant cell in Fig. 21 is ca. 13μ.



Figs. 21–22. *Saccharomyces*. Giant cells containing more than one nucleus together with normal uninucleate cells from a wort culture of the haploid strain 62–143. (× ca. 975).

As with *Lipomyces*, an attempt was made to isolate giant cells of *Saccharomyces* by means of the micromanipulator. A 2 day old culture of H 307 in wort was employed for this purpose, but it was found to be exceedingly difficult to remove a single giant cell from its chain, and therefore two whole chains were isolated in wort droplets. As controls, isolations were made of 4 normal vegetative cells. One chain, consisting of 6 giant cells, developed within 24 hours into a colony composed of both giant and normal cells, and from this colony a large cell and its bud were transferred into an empty wort droplet in the same chamber. Two days later this droplet contained a colony which again consisted of both normal and giant cells. It was transferred to wort as H 307-B. Meanwhile, all 4 controls had developed into colonies devoid of giant cells, and one of them was transferred to wort as H 307-A. After three days incubation at room temperature, H 307-A and H 307-B were examined microscopically and, surprisingly, very little difference in cellular morphology could be detected, the cells of both cultures appearing quite normal with respect to size. Six days later abundant sporulation had occurred in both H 307-A and H 307-B, and the spores were capable of germination. The cultures were also transferred to wort gelatine, and the subsequent giant colonies which developed at 15°C. were clearly dissimilar in appearance.

Additional Observations and Discussion.

The ultimate fate of a culture derived from a single giant cell is not yet known with certainty, but certain additional observations and speculations may be of interest in attempting to elucidate this problem.

In *Lipomyces* the majority of giant cells are multinucleate, and since the giant progeny of Cell C was primarily multinucleate, it is reasonable to assume that this giant cell contained more than one nucleus at the time of its isolation. Although its immediate descendants were giants, an increasing number of normal-appearing uninucleate cells made their appearance in rapidly-growing C cultures as time went on, and some six weeks later, giant cells were actually rare in cultures which had been repeatedly transferred. At the same time, 3 weeks old C cultures which had been maintained under conditions permitting only slow growth (wort gelatine at 15°C. or yeast water agar at room temperature) consisted primarily of giant cells. At the time of writing a 10 day old C culture which has been maintained on wort gelatine at 15°C. during the past two months still consists predominantly of giant cells. Measurements made on 30 of its cells give an average of 6.8 × 16.7μ, with some of them attaining the extraordinary length of 25 to 30μ. In comparison, measurements of 30 cells of a 4 day old culture growing on potato agar at room temperature average 5.7 × 7.1μ.

These observations appear to be in accordance with expectation. Although binucleate giant cells were often seen to give rise to binucleate buds (Figs. 2, 10, 12, 15), evidence for synchronized mitoses in giant cells possessing more than two nuclei was rare. Only one case was observed in which 3 nuclei appeared to be simultaneously dividing. On the other hand, many instances were noted in which a bud had fewer nuclei than its giant multinucleate mother cell (Figs. 16, 19). At present there is no evidence in this material for the existence of true polyploidy – *i.e.*, where the individual nuclei contain more than two haploid chromosome sets. If it is assumed, therefore, that polyploidy is absent and that the multinucleate condition results from supernumerary mitoses, then a uninucleate bud arising from a multinucleate giant cell should be genotypically identical with a normal uninucleate cell, and, barring mutation of nuclear disturbances, its descendants should be likewise. In this way the transformation of multinucleate giant cells into a population of uninucleate normal cells is explainable: the giant cells simply disappear, being unable to compete with the more vigorous normal cells which they themselves produce.

If this explanation is correct, a C culture of *L. lipofer* whose cells have reverted to the normal condition should be capable of normal sporulation within the course of a week on yeast water agar (Roberts 1957). A 17 day old C culture growing on potato agar was found to fulfill the criterion for reversion, its cells being small and round. It was accordingly transferred to yeast water agar and, in contrast to the non-reverted C culture previously mentioned, it produced within 6 days many characteristic outgrowths containing lightly pigmented spores which appeared normal in all respects.

This behaviour is certainly indicative of a reversion of the culture to the normal state, but it should be stressed that even when the giant cells have disappeared and sporulation is again normal, we cannot categorically state that such a reverted population is genetically identical with a normal population. The possibility that the chromosome complement of a normal uninucleate cell does actually deviate from that of a uninucleate bud cut off from a giant multinucleate cell, or that a uninucleate giant cell is genotypically different from a uninucleate normal cell cannot at present be excluded. It is unfortunate in this connection that the very low percentage of spore germination in *L. lipofer* makes it an unsuitable object for genetic studies.

With respect to *Saccharomyces*, the situation is somewhat different. The nuclear condition of the chain of giant cells isolated from H 307 was of course not known, but regardless of whether they were multinucleate or not, it seems reasonable to assume that the much more rapid reversion noted in this genus was due to its being able to propagate itself more rapidly than *Lipomyces*. Since the percentage of spore germination in *Saccharomyces* is considerably higher than in *Lipomyces*, it is possible that a detailed genetic study of the reverted progeny of its giant cells would prove fruitful in attempting to solve the problem of their identity with the normal cells. On the other hand, the very large size of the giant cells of *Lipomyces* makes them perhaps the most suitable of all yeasts for cytological investigations, particularly on the finer structure of the nucleus. Such studies have been undertaken and will be reported elsewhere.

Summary.

The spontaneous occurrence of giant cells has been observed in young cultures of *Lipomyces lipofer* and three different genotypes of *Saccharomyces*. Stained preparations of all abnormal cultures revealed that the giant cells characteristically contained more than one nucleus, the number ranging from one to six. In both genera the phenomenon was found to be transient, for rapidly-growing cultures arising from isolated giant cells reverted, at varying rates, to populations of small uninucleate cells which appeared normal in all respects.

Acknowledgement.

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STUDIES ON THE MUCOLYTIC ACTIVITY OF *VIBRIO FETUS*

by

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(Received June 30, 1958).

The mucolytic activity of hyaluronidase has been found predominantly in pathogenic organisms, mostly bacteria. The presence of hyaluronidase is considered as a sign of their stronger virulence. This ferment has the capability of decreasing the viscosity of hyaluronic acid in the body of the host so that organisms and their toxins can spread around. According to data from the literature hyaluronidase has been hitherto found in several species of bacteria: *Micrococcus pyogenes* var. *aureus* and *albus*, *Streptococcus* species from the groups A, B, C and G, *Streptococcus viridans*, *Pneumococcus* species, *Cl. perfringens* (types A, B and F), *Cl. septicum*, *Cl. novy*, *B. anthracis*, *Erysipelothrix rhusiopathiae*, *Brucella abortus*, and in some Leptospira and Treponema (2). We have recently demonstrated hyaluronidase in *Brucella suis* and *B. melitensis* (5), *Corynebacterium equi*, *C. renale*, and in *Shigella equirulis* (1).

In the protozoon *Trichomonas genitalis* we could not find hyaluronidase, but we have shown that this organism has a very active ferment which decomposes a special substratum contained in the estral mucus of cows. Therefore *Trichomonas genitalis* excretes mucinase which depolymerizes the chemically still undefined mucinous matter of the estral mucus of cows (4).

In our investigations, an account of which we are giving in this paper, we tried to find out whether *Vibrio fetus* possesses a mucinase of the hyaluronidase type, or whether this organism causing sterility in cattle has the capacity of decomposing the estral mucus of cows. Like *Trichomonas genitalis*, *Vibrio fetus* is localized in the genital organs of cattle and the infection spreads by coitus.

Our investigations were carried out with 5 strains of *Vibrio* received from the Rijksseruminrichting, Rotterdam, Holland[1]. Four of them were pathogenic strains of *Vibrio fetus* and one was an apathogenic *Vibrio* strain. Two of the pathogenic strains (No. 1 and 3) were isolated from aborted fetus of cows living in districts where vibriosis is known to occur, and the other two pathogenic strains (No. 90 and 92) were also isolated from aborted fetus, but from a district where there was no sterility in cattle. The apathogenic *Vibrio* (No. 126) was isolated from the preputium of a bull.

The organisms were cultivated in broth with 10% horse serum or 1‰ agar. For our investigations 96 hour-old cultures were used. As substratum served 1—2% potassium hyaluronate (Allen and Hanburys Ltd, London). For buffering (for pH 4.7 as an optimal pH for hyaluronidase) a solution of sodium citrate and acetic acid was added. Besides the mentioned substratum we also used natural mucine — the estral mucus of healthy cows. When working with the latter, no buffering was applied.

Viscosimetric measuring was done according to the method of WERLE *et al.* (6). The reaction mixtures were composed as follows: Into tubes of 14 mm of diameter 0.25 ml of solution of hyaluronic acid or 0.5 ml of estral mucus was introduced, to which 0.05 ml or 0.1 ml of the ferment (culture) was added. The buffer solution was added in the quantity of 0.05 ml. Immediately after that the reaction mixture was stirred with a glass rod and drawn into a capillary pipet of 0.1 ml which served as a viscosimeter. The time of running was measured by stop-watch until the mark of 0.02 or 0.01 on the viscosimeter. This measuring was carried out immediately after the reaction mixture had been stirred or 30, 60 or 120 minutes later. As controls served sterile, non-inoculated media, described above, or inactivated cultures. The inactivation was done by heating the cultures in a boiling water-bath for half an hour. The measuring was carried out at room temperature and the reaction mixtures were kept, between two consecutive measurings in a termostat at 37°C.

RESULTS.

From the results of our investigations presented in table 1 it can be realized that the changes in viscosity of the solution of

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[1] We wish to express our thanks to the Rijksseruminrichting at Rotterdam for sending cultures of the *Vibrio* strains.

TABLE 1.

No.	Substratum	Time of running of the control mixture ¹) (in seconds)	Broth culture of *Vibrio* strain No.	Time of running of the studied mixture with ferment (in seconds)	Percentage of reduction of the viscosity ²)
1	Hyaluronic acid	40.6	1	40.6	—
2	,,	36.2	3	36.8	—
3	,,	32.4	90	32.4	—
4	,,	52.0	92	50.0	3.9
5	,,	32.4	126	32.4	—
6	Estral mucus	25.2	1	8.2	67.5
7	,,	80.2	1	28.2	64.8
8	,,	∞³)	3	29.0	89.8
9	,,	51.0	3	8.4	83.5
10	,,	59.0	3	11.6	80.5
11	,,	51.0	90	12.2	76.1
12	,,	51.0	90 ⁴)	54.6	—
13	,,	59.2	90	21.0	64.5
14	,,	54.0	92	6.2	88.5
15	,,	59.2	92	8.0	86.5
16	,,	59.2	126	19.6	66.9

¹) To the substratum a sterile medium added, and in the case of hyaluronic acid, a buffer also.

²) The values indicate the changes in viscosity at 0—2 hours.

³) Mucus very dense which made measuring of the viscosity impossible. Percentage calculated after the first measuring which followed the addition of the ferment (i.e. 256.8 seconds).

⁴) Inactivated.

hyaluronic acid have been very small, a fact which suggests that the examined strains of *Vibrio* do not dispose of mucolytic activity which could depolymerize hyaluronic acid.

A completely different picture is given by the results of our studies in which hyaluronic acid has been replaced by a still undefined substratum contained in the estral mucus of cows. In using the mentioned mucus we could notice that already when diluting it the reduction of the viscosity was more pronounced than in the experiments with hyaluronic acid. For that reason the values of the reduction of viscosity in the main experiment were calculated by comparing them with the results of the controls, thus avoiding a passive reduction.

The results of our experiments, in which estral mucus was used as substratum, show that the presence of a culture causes a particularly pronounced reduction of the viscosity. Such pronounced changes can be attributed only to the activity of a mucolytic ferment which is specific for a particular substratum, contained in the estral mucus. As a proof of the presence of the ferment served the negative results obtained with the controls by using inactivated cultures of *Vibrio fetus* (see table; 12).

It should be mentioned that the effect of the ferment was noticed already at the first contact with the substratum (mucus), at the first measuring which was carried out immediately after the mixture had been stirred. This effect became more pronounced at subsequent measurings. In investigations of the mucolytic activity of *Trichomonas genitalis* (4) such an effect at the first contact with the substratum and the ferment has not always been observed.

Our observations that neither *Vibrio fetus* nor — as found in an earlier work — *Trichomonas genitalis* dispose of mucinase which would be able to decompose hyaluronic acid, but that they possess a mucinase capable of decomposing the particular substratum contained in the estral mucus of cows, are in accord with the observations of McClean (7) who has found that the hyaluronidase does not decompose the cervical mucin and that the chemical composition of hyaluronic acid should therefore be different from that of cervical mucin. It may be suggested that the mucinase which decomposes estral mucus is somewhat alike the mucinase contained in the ejaculation of the rabbit, described by Sokolovskaja (3).

Today the coital way of infection in vibriosis of cattle is considered as being almost the exclusive one. The demonstration of the specific ferment which depolymerizes the estral mucus of cattle can serve as one of the proofs for such a hypothesis. In this way the pathogenic activity of *Vibrio fetus* in the host, as well as its penetration into the uterus, can be partly explained.

Conclusion.

Vibrio fetus disposes of or can produce very active mucinase which affects the mucinous matter of the estral mucus of cows, but does not affect hyaluronic acid. The effect of the mucinase of *Vibrio fetus* is shown in a pronounced decrease of viscosity of the mentioned mucus. It is very likely that the mucinase of *Vibrio fetus* acts only in a specific substratum. Such a mucolytic activity should be taken into account when the pathogenic action of this agent is to be explained.

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UEBER VIRULENZ UND VIRULENZ-CURVEN

von

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(Eingegangen am 25. November 1958).

Die verschiedenen Lehrbücher der Bacteriologie geben die verschiedensten Definitionen des Begriffes der Virulenz. Oftmals wird die Virulenz gleichgesetzt mit der Pathogenität (z.B. JORDAN und BURROWS 1942), teilweise wird nur eine bestimmte Eigenschaft des Parasiten in Betracht gezogen, z.B. die Toxinbildung (z.B. MÜLLER 1939), die Eindringungskraft oder "invasiveness" (z.B. SCHLOSSBERGER 1952), "the degree of their disease-producing power" (z.B. ZINSSER und BAYNE-JONES 1939), "the severity of the injury created or of the invasion as a measure of the virulence" (FRANCIS 1952), "the rapidity of spread" (WILSON und MILES 1955) usw. Auf jeden Fall aber wird meistenteils Virulenz ausschliesslich auf Eigenschaften des Krankheitserregers bezogen.

Ganz anders ist die Definition für Virulenz, die DUBOS (1947) gibt:

> "Virulence is not a permanent, intrinsic property of a given species. It expresses only the ability of a given strain of the infective agent, in a certain growth phase, to produce a pathogenic state in a particular host, when introduced into that host under well defined conditions. This definition .... refers to the disease and to the host-parasite relationship, rather than to some unique attribute of the microorganism. Complete analysis of the problem of virulence would require therefore, a description of the many microbial and host factors, which affect infection and resistance".

Später weist DUBOS (1954) darauf hin, dass man Krankheit viel zu viel als einen Conflict ansieht, bei dem auf der einen Seite der Eindringer und auf der andern der Verteidigungsmechanismus stehen.

> "However the host and the parasite may be regarded as an entity, and we may think in terms of those factors which act to present the establishment of this entity".

Die Vorstellungen von Dubos ermöglichen uns folgende Definition aufzustellen:

Unter Virulenz versteht man das Ergebnis des Wechselspieles zwischen einerseits den zahlreichen bekannten (und nicht bekannten) Factoren, welche die Krankheitserregenden Eigenschaften eines bestimmten Stammes eines Microorganismus oder anderen Parasiten für einen bestimmten Wirt ausmachen, und andererseits den zahlreichen bekannten (und nicht bekannten) Factoren, welche die Empfänglichkeit und die Resistenz dieses Wirtes bestimmen. Hierher gehören auch alle Erscheinungen der unspecifischen Immunität. Zwischen beiden Factorengruppen wird das Erreichen eines bestimmten Gleichgewichtes angestrebt.

Die Bestimmung der Virulenz wird auf verschiedene Weisen durchgeführt. Die experimentelle Feststellung der "Dosis letalis minima", also der kleinsten Menge von Erregern, welche ein empfängliches Tier grade eben tötet, ist ein vollkommen ungeeigneter Massstab. Es liegt ja keine einfache chemische Reaction vor, sondern es handelt sich um das Wechselspiel von zwei ursprünglich voneinander unabhängigen physiologischen Systemen.

Schon besser ist nach Pondman (1957) die Berechnung der MLD_{50}, also die Angabe der Menge des Erregers, die nach der Infection einer grösseren, möglichst gleichmässig zusammengesetzten, Serie von Versuchstieren grade 50 % tötlich erkranken lässt. Die Incubationszeit und die Länge des Krankheitsprocesses wird hierbei nicht ausgedrückt. Bei der Berechnung der MLD_{50} wird nur der Endzustand des biologischen Geschehens berücksichtigt, nicht aber die Tatsache, dass es sich bei der Virulenz um einen durchaus dynamischen Vorgang handelt, um ein Geschehen, das in der Zeit verläuft, bis endlich der eben erwähnte Endzustand erreicht worden ist. Graphisch dargestellt gibt die MLD_{50} einen Punkt an, keine Curve.

Gerne macht man auch Gebrauch von der Methode von Gard (1940). Er geht bei seiner Methode zur Messung der Virus-Activität aus von der Gleichung

$$\frac{1}{T} = \frac{1/t_1 + 1/t_2 + 1/t_3 \ldots . + 1/t_n}{N} = \frac{\Sigma 1/t}{N}.$$

Er benutzt also den reziproken Wert der harmonischen Mittelwerte der Incubationsperioden der einzelnen Infectionsdosen. Diese Werte werden gegenüber der log-Concentration des Virus (in seinem

TABELLE 1 (nach GARD).

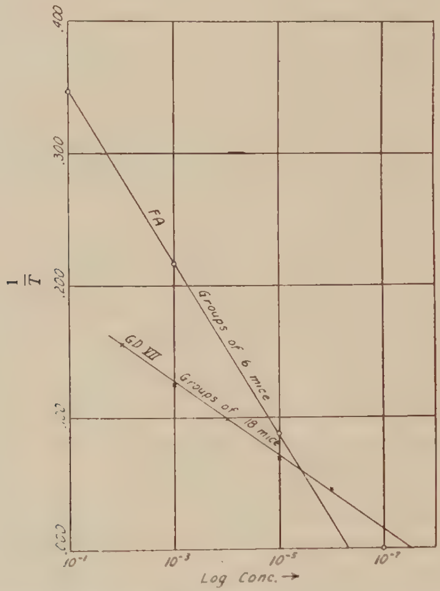
Titration Results Obtained with Two Different Strains of Mouse Encephalomyelitis Virus*

Strain	Concentration of brain	N No. inoculated	n No. sick	No. of mice becoming sick after t days																			Average incubation time *days*	$\frac{\Sigma 1/t}{N}$
				2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	**		
GD VII	10^{-2}	18	18			2		5	6	4	1												6.7	0.156
GD VII	10^{-3}	18	18				2	1	2	6	1	3	1	1			1						8.6	0.125
GD VII	10^{-4}	18	18					2	1	1		2	4	4	2	1	1						10.8	0.099
GD VII	10^{-5}	17	15									1	3	4	1	1	2		3			2	13.3	0.069
GD VII	10^{-6}	18	12							1					2	1			6	1	1	6	15.6	0.045
FA	10^{-1}	6	6	1	4	1																	3.0	0.347
FA	10^{-3}	6	6			2	4																4.7	0.217
FA	10^{-5}	6	4				1		1			1						1				2	9.5	0.088
FA	10^{-7}	6	0																			6	—	0.000

* Dose = 0.03 cc.

** Number lived.

Falle feuchtes Mäusegehirn) eingetragen. Es entsteht eine annähernd lineare Curve. Bei dieser Methode von GARD wird die Incubationsperiode berücksichtigt, ebenso auch die Virus-Concentration. Die beiden Beispiele von GARD sind in Tabelle 1 und Graphik 1 wiedergegeben.



Graphik 1. Linear relationship between the reciprocal of the harmonic mean of the incubation periods ($1/T$) and the logarithm of the concentration of virus (nach GARD).

Aber auch diese Methode gibt nur Endzustände an, während die Dynamik des zeitlichen Geschehens nicht zum Ausdruck gebracht wird.

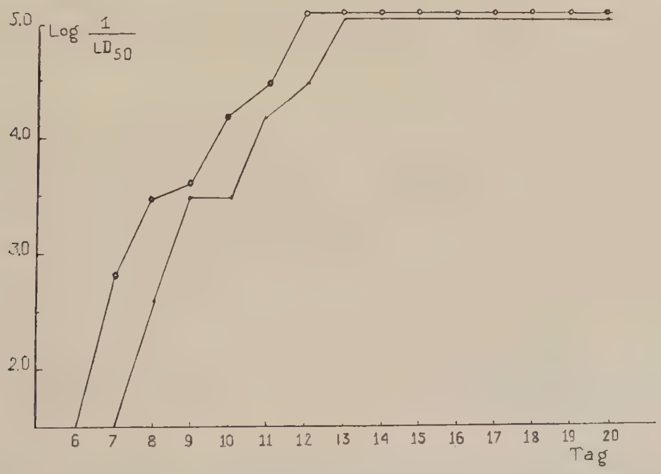
Und doch dürfte es möglich sein, sich eine Vorstellung von der Virulenz zu machen, wenn man eine in der Zeit verlaufende Kurve betrachtet. Hierbei brauchen die zahlreichen Factoren der beiden Parteien nicht näher analysiert zu werden.

Um dies deutlich zu machen, sei von einem Versuch ausgegangen, in dem Versuchstiere einer möglichst gleichmässig zusammengesetzten Population mit fallenden Verdünnungen einer ebenfalls möglichst gleichmässigen Erregerpopulation so inficiert werden, dass (am liebsten) in den niedrigen Verdünnungsgraden alle Versuchstiere erkranken oder sterben und in den höchsten Verdünnungsgraden (am liebsten) alle Versuchstiere gesund und am Leben bleiben. Die inficierten Tiere werden beobachtet bis keine neuen Erkrankungs- oder Todesfälle vorkommen. Dann wird von dem täglichen Status des Gesamtversuches die LD_{50} nach Reed und Munch berechnet.

Als Beispiel sei die Bestimmung der Virulenz von Gelbfiebervirus aus dem Gehirn einer Maus wiedergegeben, die mit dem Vaccinstamm 17 D intracerebral inficiert war und am 12. Tage deutlich gelähmt war. Mit der in Rinderalbumin hergestellten Gehirnsuspension in den Verdünnungen von 1/100 – 1/1,000,000 wurden je 6 Mäuse mit je 0.02 ml intracerebral inficiert, wobei es sich um den durch strenge Inzucht (Bruder-Schwester-Kreuzung) weitergezüchteten Stamm von Swiss-Mäusen, Unterstamm Utrecht, handelte. Hierbei ergab sich folgendes Resultat, das in Tabelle 2 (S. 118/119) wiedergegeben ist.

Bei dieser Methode sind alle wirksamen Factoren zur Geltung gekommen: In den niedrigen Verdünnungen vor allem die Factoren des Erregers, die schädlichen Einfluss auf den Wirt ausüben, in den höheren Verdünnungen vor allem die Factoren des Wirtes, die schädlichen Einfluss auf den Erreger auszuüben vermögen. Die einzelnen Factoren brauchen nicht bekannt zu sein.

Von den an jedem Tag toten Mäusen wird nun nach Reed und Munch die LD_{50} berechnet, wobei nicht von den täglich beob-



Graphik 2. Virulenzkurve von Stamm 17D, zweite Gehirnpassage 0.02 ml cerebral. ○——○ Erkrankung; ●——● Tod.

TABEL

Ursprüngliches Protokoll des Versuches, Mäuse mit der 1. Passage des 17 I

Infection	Mit Gehirn v. Maus 58737, die mit 17D 1/4096 intracerebral inficier																	
Infections-Dosis	10^{-2}						10^{-3}						10^{-4}					
Tier No.: inficiert am 30-7-58	1	2	3	4	5	6	1	2	3	4	5	6	1	2	3	4	5	6
1 Tag	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
2 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
3 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
4 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
5 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
6 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
7 ,,	g	g	g	g	g	,,	g	g	g	,,	,,	,,	,,	,,	,,	,,	,,	,,
8 ,,	T	T	T	T	T	g	T	T	g	g	g	g	,,	,,	,,	,,	,,	,,
9 ,,						T			T	T	T	T	g	,,	,,	,,	,,	,,
10 ,,													g	g	g	,,	,,	,,
11 ,,													T	T	T	g	,,	,,
12 ,,																T	g	,,
13 ,,																	T	,,
14 ,,																		,,
15 ,,																		,,
16 ,,																		,,
17 ,,																		,,
18 ,,																		,,
19 ,,																		L

L = lebt, g = gelähmt. T = tot.

achteten frischen Todesfällen der einzelnen Gruppen ausgegangen wird, sondern von den cumulierten Todesfällen der Gruppen bis zu diesem Tage. Man erhält auf diese Weise folgende Uebersicht des Versuches, die in Tabelle 3 (S. 120) wiedergegeben ist. Hierin ist für die verschiedenen Tage die LD_{50} berechnet und die reziproken Werte der dazu gehörigen Logarithmen.

Um einen Vergleich zu bekommen, wurde in diesem Falle auch die gleiche Berechnung für den Beginn der Krankheit durchgeführt.

Bei der graphischen Darstellung wurden auf der Abscisse die Tage und auf der Ordinate die Logarithmen der reziproken Werte der LD_{50} und der PD_{50} (= paralys. Dosis von 50%) eingetragen. Die Ergebnisse finden sich in der Graphik 2 (S. 117).

2.

us zu inficieren, nebst Berechnung der LD_{50}-werte und PD_{50}-werte.

war. Infections-dosis 0.02 ml cerebral.												LD_{50}		PD_{50}	
10^{-5}						10^{-6}						$\frac{1}{LD_{50}}$	log.	$\frac{1}{PD_{50}}$	log.
1	2	3	4	5	6	1	2	3	4	5	6				
L	L	L	L	L	L	L	L	L	L	L	L				
,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,				
,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,				
,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,				
,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,				
,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,				
,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,			700	2.8
,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	440	2.6	3,200	3.5
,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	3,200	3.5	4,000	3.6
g	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	3,200	3.5	14,500	4.2
T	g	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	14,500	4.2	32,000	4.5
	T	g	g	,,	,,	,,	,,	,,	,,	,,	,,	32,000	4.5	132,000	5.1
		T	T	,,	,,	,,	,,	,,	,,	,,	,,	132,000	5.1	,,	,,
				,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
				,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
				,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
				,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
				,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
				L	L	L	L	L	L	L	L	132,000	5.1	132,000	5.1

Es sei darauf hingewiesen, dass hier, wie in allen weiteren Beispielen an Hand von Gelbfieber-Versuchen, die Berechnungen basiert sind auf der cerebral injicierten Dosis von 0.02 ml der verschiedenen Verdünnungen. Soll die Angabe "per ml" erfolgen, so müssen die Werte für PD_{50} und LD_{50} mit 50 multipliziert werden.

Beide Kurven laufen zuerst ziemlich parallel, um schliesslich einander zu decken. Die aus den PD_{50}-Werten gebildete Kurve liegt zeitlich vor der LD_{50}-Kurve. In beiden Fällen aber zeigen die Kurven drei Merkmale, durch welche die Virulenz characterisiert werden kann.

Zunächst zeigt sich bei der PD_{50}-Kurve die Länge der Incubationsperiode und bei der LD_{50}-Kurve die Länge

TABELLE 3.

Berechnung der LD_{50}-Werte und PD_{50}-Werte im verkürzten Protokoll (vgl. Tabelle 2).

Infections-Dosis	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	$\frac{1}{LD_{50}}$	log.	$\frac{1}{PD_{50}}$	log.
1 Tag	0/6*	0/6	0/6	0/6	0/6				
2 ,,	,,	,,	,,	,,	,,				
3 ,,	,,	,,	,,	,,	,,				
4 ,,	,,	,,	,,	,,	,,				
5 ,,	,,	,,	,,	,,	,,				
6 ,,	,,	,,	,,	,,	,,				
7 ,,	,,	,,	,,	,,	,,			700	2.8
8 ,,	5/6	2/6	,,	,,	,,	440	2.6	3,200	3.5
9 ,,	6/6	6/6	,,	,,	,,	3,200	3.5	4,000	3.6
10 ,,	,,	,,	,,	,,	,,	3,200	3.5	14,500	4.2
11 ,,	,,	,,	3/6	1/6	,,	14,500	4.2	32,000	4.5
12 ,,	,,	,,	4/6	2/6	,,	32,000	4.5	132,000	5.1
13 ,,	,,	,,	5/6	4/6	,,	132,000	5.1	,,	,,
14 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
15 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
16 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
17 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
18 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,
19 ,,	6/6	6/6	5/6	4/6	0/6	132,000	5.1	132,000	5.1

* 0/6 = 0 tot von 6.

des Gesamt-Krankheitsverlaufes (Incubationsperiode + Dauer des manifesten Krankheitsverlaufes), ausgedrückt in Tagen vom Beginn der erfolgte Infection an bis zu einem Termin, der es möglich macht, den ersten Wert von PD_{50} bzw. LD_{50} zu berechnen. Es geht also nicht um den Zeitpunkt der ersten Lähmungsfälle bzw. der ersten Todesfälle, sondern um den Zeitpunkt, dass man zum ersten Mal von einer PD_{50} bzw. LD_{50} sprechen kann. Dies ist naturgemäss erst dann möglich, wenn eine bestimmte Anzahl von Versuchstieren, abhängig von der Anzahl der Individuen innerhalb jeder Gruppe, erkrankt bzw. gestorben ist. Die Gesamtzahl der erkrankten bzw. gestorbenen Tiere im Gesamtversuch muss mindestens gleich sein der Anzahl der gesund gebliebenen bzw. überlebenden Individuen der stärksten Infectionsdosis. Hierbei muss man natürlich bedenken, dass bis zu einem bestimmten Grade die Länge der Incubationsperiode bwz. des Gesamt-Krankheitsverlaufes von dem absoluten Wert der stärksten Infectionsdosis abhängt. Wenn man annimmt, dass innerhalb jeder Versuchstier-Gruppe 6 Mäuse gebraucht werden, dann ist die erste exacte Berechnung der PD_{50} bezw. LD_{50} möglich, wenn folgende Bedingungen erfüllt sind:

Minimum von erkrankten bzw. gestorbenen und gesunden bzw. lebenden Versuchstieren, um die Incubationsperiode bzw. Gesamt-Krankheitsdauer zu berechnen.

Gesund gebliebene bzw. überlebende Tiere der stärksten Infectionsdosis:	erkrankte bzw. gestorbene Tiere der stärksten Infectionsdosis:	erkrankte bzw. gestorbene Tiere von den weiteren Infectionsdosen:
3	3	
4	2	2
5	1	4
6	0	6

Ausser der Länge der Incubationsperiode bzw. des Gesamt-Krankheitsverlaufes ergibt sich aber noch ein Merkmal der Virulenz-Curven, nämlich eine bestimmte Steilheit des Anstieges. Dieser kann sehr langsam oder aber sehr schnell vor sich gehen, bis ein weiters Ansteigen aufhört, also ein Maximum erreicht wird. Man kann hier vielleicht von einer Verlaufs-Schnellheit oder Anstiegschnellheit sprechen. Dies ist der Zeitverlauf vom Termin an, der es möglich macht, den ersten Wert für PD_{50} bzw. LD_{50} zu berechnen, bis zu dem Zeitpunkt, dass keine weiteren Erkrankungen bzw. keine weiteren Todesfälle innerhalb des Beobachtungstermins mehr stattfinden.

Endlich kann als drittes Merkmal der Virulenz-Curven die absolute Höhe der Virulenz dienen, also der Punkt, an dem das Maximum an Erkrankungen bzw. an Todesfällen erreicht worden ist und in keiner der verschieden stark inficierten Gruppen ein einziger Erkrankungsfall bzw. Todesfall mehr vorkommt. Dieser Punkt wird in der Praxis in der Regel dann berechnet, wenn einfach die PD_{50} oder die LD_{50} am Ende des Versuches festgestellt wird.

Bei den Virulenzkurven sind also drei deutliche Merkmale zu unterscheiden, nämlich: die Incubationsperiode bzw. Gesamt-Krankheitsdauer; Verlauf- oder Anstiegschnellheit und absolute Höhe der Virulenz. Alle diese drei Merkmale werden sowohl durch die Factoren der Pathogenität des Parasiten als auch durch die Empfänglichkeit und Resistenz des Wirtes beeinflusst. Dies drückt sich deutlich in den entstehenden Curven aus.

Es ist erwünscht, die Werte von GARD in Tabelle 1 zu benutzen, um die hier vorgeschlagene Virulenz-Kurve auszuführen. In Tabelle 4 ist die Berechnung wiedergegeben und in Graphik 3 die hieraus entstehende Curve. Deutlich treten hier die Unterschiede zwischen den beiden Stämmen hervor und zwar in der Darstellung der Incubationsperiode aber dann auch in der Verlaufschnellheit.

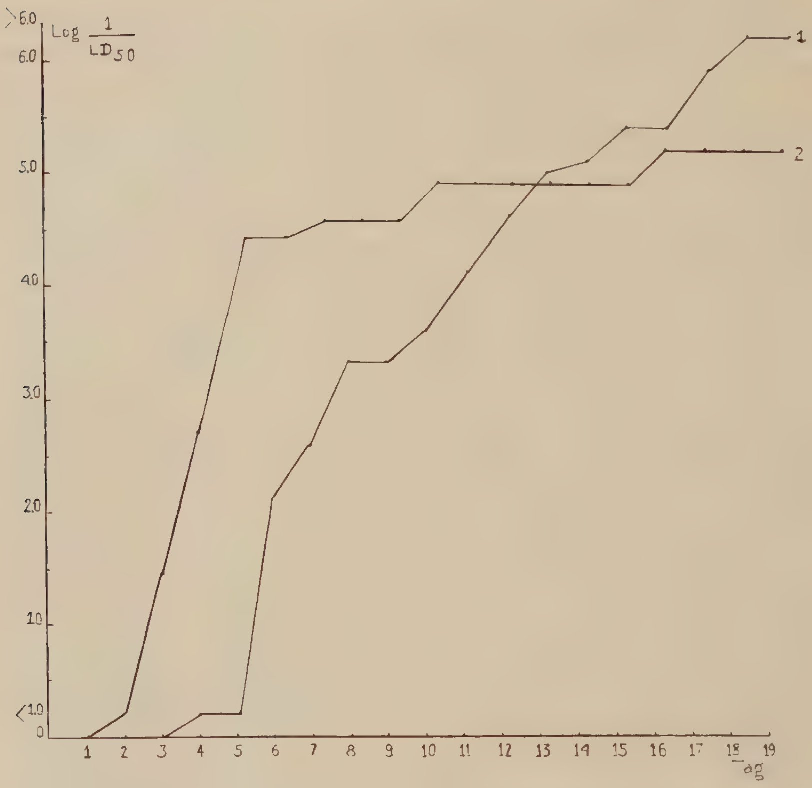
Im Nachfolgenden seien noch einige Beispiele gegeben, aus denen der Gebrauch der Virulenzcurven deutlich wird, und aus denen zu ersehen ist, wie durch Beeinflussung eines einzelnen Factors der Verlauf der Curve geändert wird. Es steht dem Beobachter frei, von den Erkrankungen oder von den Todesfällen der Versuchstiere auszugehen. Alle nunmehr folgenden eigenen Versuche sind mit Gelbfieberstämmen ausgeführt, wobei es zweckmässiger erscheint, von den Todesfällen auszugehen. Bei dem Vaccinstamm 17D kommt es zwar meist schon 1–2 Tagen vor dem Tode zu deutlich erkennbaren Krankheitserscheinungen (Lähmungen), so dass hier sowohl die Erkrankung als auch der Tod gleichmässig brauchbare Werte ergeben würde. Die neurotropen Gelbfieberstämme aber verhalten sich insofern anders, als häufig das Lähmungs-Stadium nur wenige Stunden dauert, sodass man sehr häufig Todesfälle sieht, bei denen es nicht möglich war, das kurzdauernde Lähmungsstadium zu beobachten. Bei den neurotropen Stämmen ist also unbedingt der

TABELLE 4.

Berechnung der verschiedenen PD_{50}-Werte der Mäuse-Encephalomyelitistiere von GARD.

Stamm	GD VII							FA					
Infections-Dosis	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	$\frac{1}{PD_{50}}$	log.	10^{-1}	10^{-3}	10^{-5}	10^{-7}	$\frac{1}{PD_{50}}$	log.
2 Tag								1/6				<10	
3 ,,								5/6				26	1.4
4 ,,	2/18*					<100		6/6	2/6			560	2.7
5 ,,	,,	2/18				<100		,,	6/6	1/6		40,000	4.6
6 ,,	7/18	3/18	2/18			137	2.1	,,	,,	,,		,,	,,
7 ,,	13/18	5/18	3/18			410	2.6	,,	,,	2/6		56,000	4.7
8 ,,	17/18	11/18	4/18		1/18	2,255	3.4	,,	,,	,,		,,	,,
9 ,,	18/18	12/18	,,		,,	2,800	3.4	,,	,,	,,		,,	,,
10 ,,	,,	15/18	6/18	1/17	,,	5,100	3.7	,,	,,	3/6		100,000	5.0
11 ,,	,,	16/18	10/18	4/17	,,	17,500	4.2	,,	,,	,,		,,	,,
12 ,,	,,	17/18	14/18	8/17	,,	56,000	4.7	,,	,,	,,		,,	,,
13 ,,	,,	,,	16/18	9/17	3/18	112,500	5.1	,,	,,	,,		,,	,,
14 ,,	,,	,,	17/18	10/17	4/18	175,000	5.2	,,	,,	,,		,,	,,
15 ,,	,,	18/18	18/18	12/17	,,	285,000	5.5	,,	,,	,,		,,	,,
16 ,,	,,	,,	,,	,,	,,	,,	,,	,,	,,	4/6		180,000	5.3
17 ,,	,,	,,	,,	15/17	10/18	1000,000	6.0	,,	,,	,,		,,	,,
18 ,,	,,	,,	,,	,,	11/18	>1000,000	>6.0	,,	,,	,,		,,	,,
19 ,,	18/18	18/18	18/18	15/17	12/18	>1000,000	>6.0	6/6	6/6	4/6	0/6	180,000	5.3

* 2/18 = 2 tot von 18.

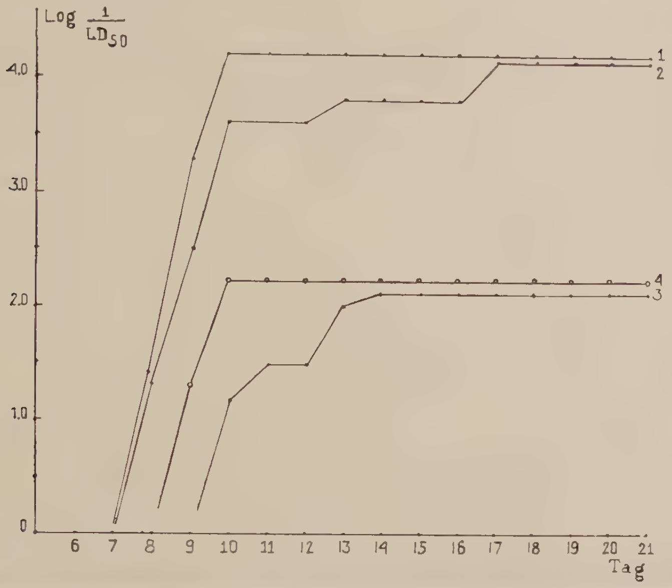


Graphik 3. Virulenzcurve nach den Werten von Gard.
1 = Stamm GD VII.
2 = Stamm FA.

eintretende Tod der eintretende Krankheit als Grundlage der Berechnung vorzuziehen. Und um gleichmässige Resultate zu ererhalten, wurde bei allen Gelbfieberversuchen der eingetretene Tod als Berechnungsbasis gewählt.

Zunächst sei ein Versuch wiedergegeben, in dem der Vaccinstamm 17D in 1 % Rinderalbuminauflösung (Fraction 5) suspendiert wurde, worauf die Verdünnung in Zehnerpotenzen erfolgte. Die Verdünnungsröhrchen wurden in Eiswasser gebracht, und mit je 6 Mäusen für jede Verdünnung wurde direct nach 4 Stunden und nach 24 Stunden eine Virulenzeinstellung vorgenommen. Die errechneten Werte sind in der Graphik 4 als Kurven nebeneinander gestellt.

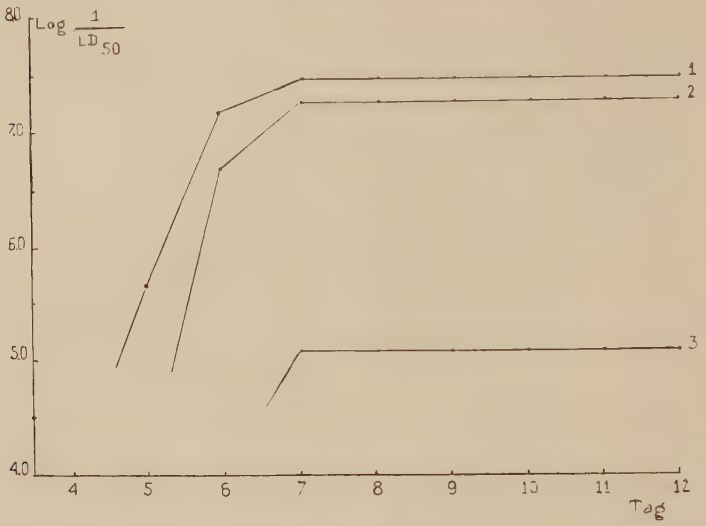
Es zeigt sich hierbei deutlich der Einfluss des Suspensionsmittel



Graphik 4. Virulenzcurven: Einfluss des Suspensionsmittels im Laufe der Zeit auf den Stamm 17D in 1% Rinderalbumin, 0.02 ml cerebral.
1 = directe Injection.
2 = Injection nach 4 Stunden bei 0°C.
3 = Injection nach 24 Stunden bei 0°C.
4 = Theoretische Curve unter Annahme, dass direct das Virus nur 1% der wirklichen Menge betragen würde.

im Verlaufe der Zeit auf die Virulenz: Bereits nach 4 Stunden hat die Verlaufschnellheit etwas abgenommen, wenn auch die Gesamtkrankheitsdauer und die absolute Höhe der Virulenz unverändert geblieben ist. Nach 24 Stunden aber zeigt sich sehr deutlich der deletäre Einfluss des Suspendierungsmittels auf die Virulenz, denn alle Merkmale der Curve sind beeinflusst: Die Gesamtkrankheitsdauer is verlängert, die Verlaufschnellheit hat sich verlangsamt, aber vor allem die absolute Höhe der Virulenz ist ansehnlich gesunken. Diese Aenderung der Virulenzkurve ist zweifellos auf Absterbe-Erscheinungen des Virus im Suspensionsmittel zurückzuführen, denn wenn man eine theoretische Kurve annimmt, die von der Annahme ausgeht, dass in jedem Röhrchen nur 1% der ursprünglichen Virusmenge übrig geblieben ist, so erhält man eine Kurve, welche sehr an die Virulenzkurve erinnert, die mit dem 24 Stunden lang bei 0°C. bewahrtem Material erhalten worden ist.

Dass es sich bei den soeben geschilderten Veränderungen der Viru-

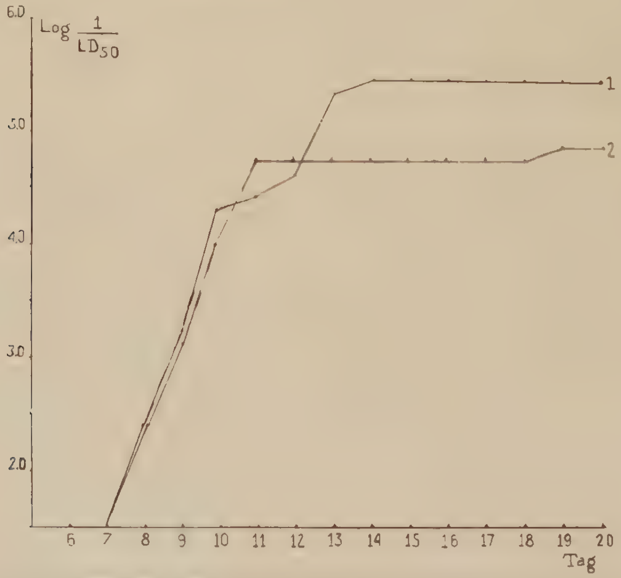


Graphik 5. Virulenzcurve: Einfluss des Suspensionsmittels im Laufe der Zeit auf den neurotropen Asibi-stamm in 1% Rinderalbumin, 0.02 ml cerebral.

1 = directe Injection.

2 = Injection nach 3½ Stunden bei 0°C.

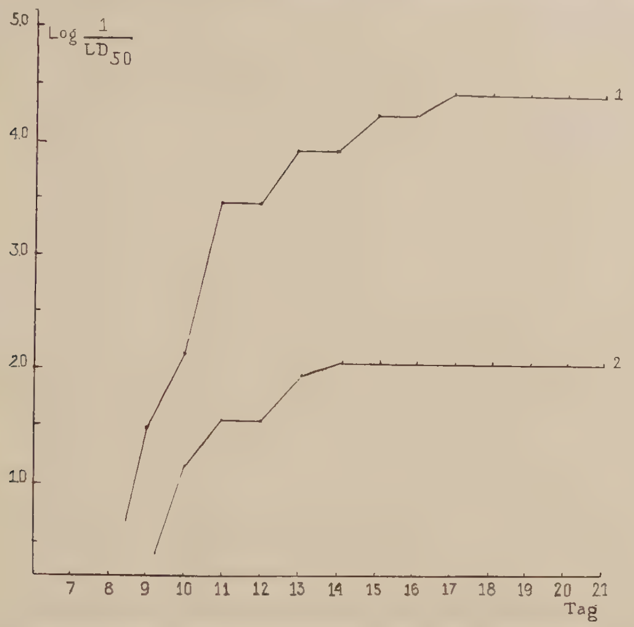
3 = Injection nach 24 Stunden bei 0°C.



Graphik 6. Einfluss des Suspensionsmittels auf Stamm 17D, verimpft nach 3½ Stunden bei 0°C.

1 = 10% natives Mäusegehirn in NaCl.

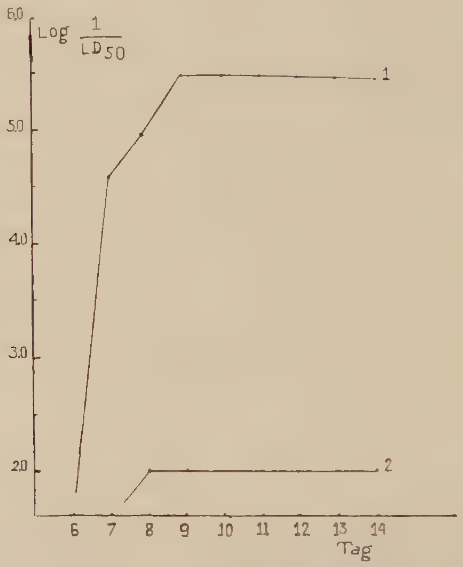
2 = 10% normales Menschenserum in NaCl.



Graphik 7. Einfluss des Suspensionsmittels auf den Stamm 17D, verimpft nach 24 Stunden bei 0°C.
1 = 10% natives Mäusegehirn in NaCl.
2 = 1% Rinderalbumin in NaCl.

lenzkurven im Verlaufe der Zeit nicht um zufällige Eigentümlichkeiten des Stammes 17D handelt, ist aus Graphik 5 deutlich zu erkennen. Hier wurde der gleiche Versuch mit dem neurotropen Asibi-stamm durchgeführt, der direct, nach 3½ und nach 24 Stunden verimpft wurde. Nach 3½ Stunden ist nur eine geringe Beeinflussung der Virulenzkurve zu sehen, die aber nach 24 Stunden sehr deutlich geworden ist, und die vollkommen an die Curve in Graphik 4 erinnert.

Sehr zweckmässig erweist sich die Ausarbeitung der Virulenzcurven bei der Untersuchung der Eignung der verschiedenen Suspendierungsmittel des Gelbfiebervirus. In Graphik 6 findet sich ein Vergleich zwischen 10 % normal Menschenserum in physiologischer Kochsalzlösung und 10 % nativem Normal-Mäusegehirn in phys. Kochsalzlösung als Suspendierungsmittel für das Virus 17D nach 3½ Stunden Aufenthalt in Eiswasser. Ebenso ist in Graphik 7 ein Vergleich dargestellt zwischen 1 % Rinderalbumin (Fraction 5) in phys. Kochsalzlösung und 10 % Normal-Mäusegehirn in phys. Kochsalzlösung als Suspendierungsmittel für das Virus 17D nach

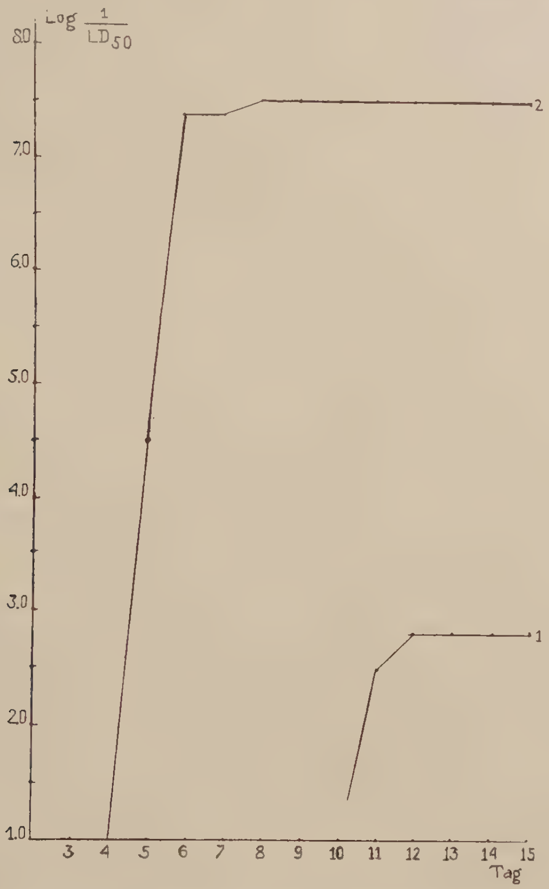


Graphik 8. Einfluss des Suspensionsmittels auf den neurotropen 17D-Stamm, verimpft nach 24 Stunden bei 0°C.
1 = 10% natives Mäusegehirn in NaCl.
2 = 1% Rinderalbumin in NaCl.

24 Stunden Aufenthalt in Eiswasser. Schliesslich zeigt Graphik 8 die Virulenzkurven für den neurotropen 17D-Stamm, über den bereits Collier, de Roever-Bonnet und Hoekstra (1959) berichtet haben, in Rinderalbumin und Mäusegehirn, wo sich prinzipiell die gleichen Unterschiede zeigen, die sich auch bei dem Stamm 17D ergeben haben. In beiden Fällen ergibt sich sehr deutlich, dass Gelbfiebervirus in einer 10 %igen Suspension von Mäusegehirn nach 24stündigem Aufenthalt in Eiswasser van 0°C. eine erheblich höhere Virulenz aufweist als in 1 %igen Rinderalbumin (d.h. bessere Möglichkeiten findet, um am Leben zu bleiben).

Die bisherigen Beispiele haben Factoren beleuchtet, die mit dem Parasiten in Zusammenhang stehen. Aber auch die Factoren des Wirtes beeinflussen tiefgehend die Virulenzkurven. Im nachfolgenden seien als Beispiel nur Versuche mitgeteilt, die den Einfluss der activen Immunität auf die Virulenz erkennen lassen.

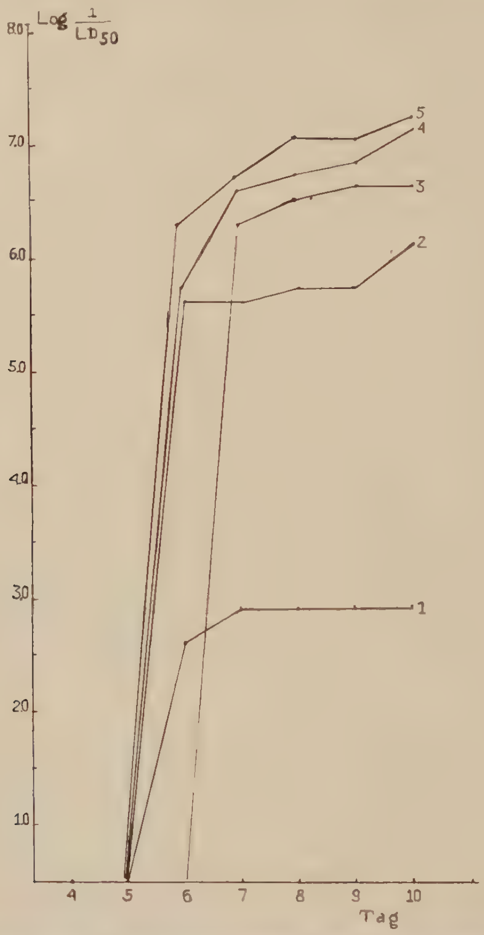
In Graphik 9 findet sich eine Gegenüberstellung einer Virulenzcurve von 6-mal mit dem neurotropen Asibi-stamm mit einer Dosis von 0.5 ml 1/200 immunisierten Mäusen mit der Virulenzcurve der



Graphik 9. Einfluss der intraperitonealen Immunisierung mit dem neurotropen Asibi-stamm auf die Infection mit dem homologen Stamm.
1 = intraperitoneale Immunisierung mit dem neurotropen Asibi-stamm 0.5 ml 1/200 6×.
2 = normale Controllmäuse.

entsprechenden normalen Controll-Mäusen nach Infection mit dem homologen Stamm.

Weiterhin zeigt Graphik 10 die Virulenzcurven von 1-mal, 2-mal und 5-mal intraperitoneal mit dem Stamm 17D immunisierten Mäusen im Vergleich mit den Virulenzcurven cerebral 1-mal immunisierter und normaler Controll-Mäuse nach Infection mit dem neurotropen Asibi-stamm. Die bei intraperitonealer Vorbehandlung schwache Immunisierungskraft des Stammes 17D für die mit dem neurotropen Asibi-stamm nachinficierte Maus wird hier sehr deut-

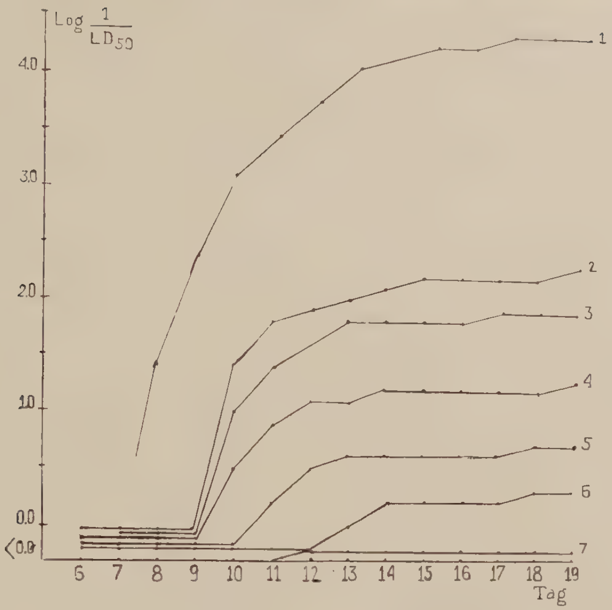


Graphik 10. Einfluss der intraperitonealen bzw. intracerebralen Immunisierung mit dem 17D-Stamm auf die cerebrale Infection mit dem neurotropen Asibi-stamm.

1 = cerebrale Immunisierung mit 17D 0.02 1/4096.
2 = intraperitoneale Immunisierung mit 17D 0.5 1/50 5 ×.
3 = intraperitoneale Immunisierung mit 17D 0.5 1/10 2×.
4 = intraperitoneale Immunisierung mit 17D 0.5 1/10 1×.
5 = normale Mäuse als Controllen.

lich, worauf schon COLLIER, DE ROEVER-BONNET und HOEKSTRA (1958) hingewiesen hatten.

Besonders interessant sind aber Versuche, die mit 2617 immunisierten Mäusen und mit 1437 normalen Controll-mäusen durchgeführt wurden. Die Immunisierung erfolgte durch intracerebrale In-

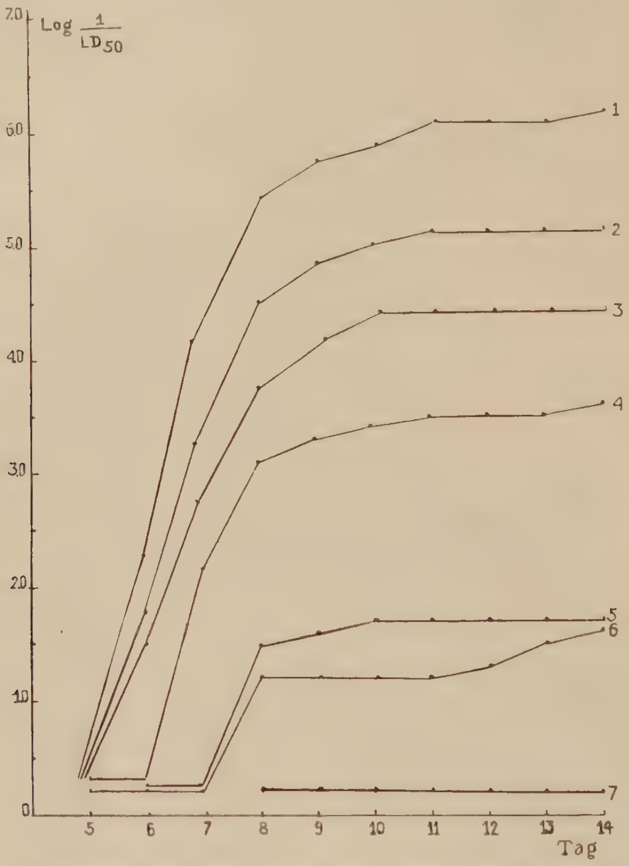


Graphik 11. Einfluss der cerebralen Immunisierung mit dem 17D-Stamm auf die cerebrale Infection mit dem homologen Stamm. Infection mit dem 17D Stamm 0.02 ml cerebral.

1 = normale Controllmäuse (441 Tiere).
2 = Immunisierung mit 17D 1/262144 0.02 ml cerebral (218 Tiere).
3 = Immunisierung mit 17D 1/65536 0.02 ml cerebral (205 Tiere).
4 = Immunisierung mit 17D 1/16348 0.02 ml cerebral (162 Tiere).
5 = Immunisierung mit 17D 1/4096 0.02 ml cerebral (115 Tiere).
6 = Immunisierung mit 17D 1/1024 0.02 ml cerebral (79 Tiere).
7 = Immunisierung mit 17D 1/256 0.02 ml cerebral (56 Tiere).

fection mit dem Vaccinestamm 17D in einer Menge von 0.02 ml und zwar in den Concentrationen von 1/256, 1/1024, 1/4096, 1/16384, 1/65536 und 1/262144. Die 2617 überlebenden Tiere wurden in drei Gruppen verteilt. Die erste Gruppe wurde mit verschiedenen Zehnerpotenzen des Vaccinstammes (beginnend mit 1/1) intracerebral inficiert, die zweite Gruppe mit dem neurotropen 17D-Stamm und die dritte Gruppe mit dem neurotropen Asibi-stamm (in beiden Fällen beginnend mit 1/10). Die Versuche erstrecken sich über etwa 8 Monate und wurden schliesslich zusammengefasst. Die Virulenzcurven sind in den Graphiken 11–13 wiedergegeben.

Alle drei Versuchsgruppen ergeben das gleiche Resultat, dass nämlich die intracerebrale Immunisierung mit selbst den minimalen Dosen von 0.02 ml von 1/262144 des Stammes 17D imstande ist,

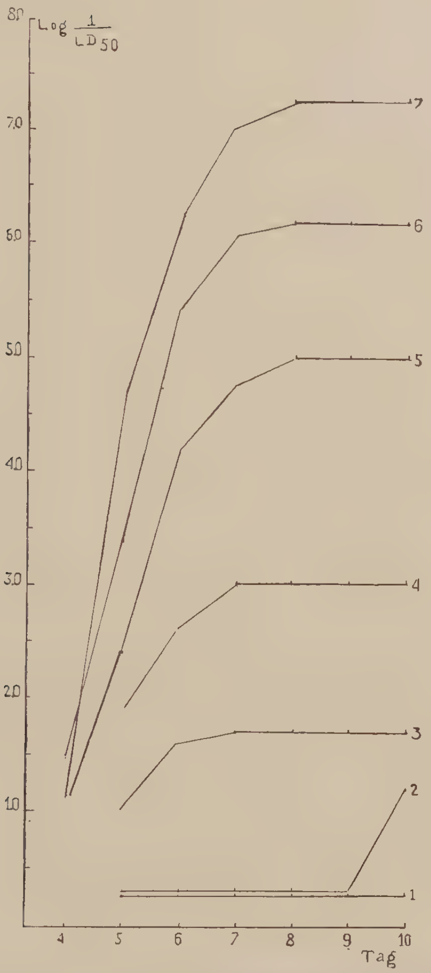


Graphik 12. Einfluss der cerebralen Immunisierung mit dem 17D-Stamm auf die cerebrale Infection mit dem neurotropen 17D-Stamm. Infection 0.02 ml cerebral.

1 = normale Controllmäuse (554 Mäuse).
2 = Immunisierung mit 17D 1/262144 0.02 ml cerebral (283 Tiere).
3 = Immunisierung mit 17D 1/65536 0.02 ml cerebral (221 Tiere).
4 = Immunisierung mit 17D 1/16348 0.02 ml cerebral (156 Tiere).
5 = Immunisierung mit 17D 1/4096 0.02 ml cerebral (113 Tiere).
6 = Immunisierung mit 17D 1/1024 0.02 ml cerebral (87 Tiere).
7 = Immunisierung mit 17D 1/256 0.02 ml cerebral (41 Tiere).

eine deutliche Immunität gegen den homologen Stamm und gegen zwei neurotrope Gelbfieberstämme zu erzeugen. Je höher die immunisierende Dosis, desto stärker auch die Immunität.

Bei einem constant bleibenden Microorganismus und einer constanten Structur der Wirtspecies werden sich bei der gleichen In-



Graphik 13. Einfluss der cerebralen Immunisierung mit dem 17D-Stamm auf die cerebrale Infection mit dem neurotropen Asibi-stamm. Infection 0.02 ml cerebral.

1 = immunisiert mit 17D 1/256 0.02 cerebral (= 22 Tiere).
2 = immunisiert mit 17D 1/1024 0.02 cerebral (= 65 Tiere).
3 = immunisiert mit 17D 1/4096 0.02 cerebral (=106 Tiere).
4 = immunisiert mit 17D 1/16384 0.02 cerebral (=141 Tiere).
5 = immunisiert mit 17D 1/65536 0.02 cerebral (=227 Tiere).
6 = immunisiert mit 17D 1/262144 0.02 cerebral (=310 Tiere).
7 = normale Controllmäuse (=442 Tiere).

fectionsweise im Princip auch stets die gleichen Virulenzcurven ergeben.

Ganz anders aber ist es, wenn einer der Factoren verändert wird, beispielweise die Structur der Wirtspecies. Hierbei braucht die Veränderung keineswegs genetisch bedingt zu sein.

Es ist möglich mittels der Virulenzcurve eindeutig zu zeigen, dass auch die geringfügigste Aenderung der Immunitätslage der Wirtspecies sich in der Virulenzcurve wiederspiegelt.

Auch bei der specifischen activen und passiven Immunität spielen sowohl Wirt wie Parasit eine gleichmässig wichtige Rolle, die sich schliesslich ebenfalls in der Virulenz ausdrückt. Virulenz ist ein volkommen dynamischer Begriff.

Sowohl Parasit als auch Wirt sind beide lebende physiologische Systeme, die einander gegenüber stehen. Wohl nur in seltenen Fällen finden wir einen "efficient parasite", der nach SWELLENGREBEL (1939) "lives in harmony with its host, nursing upon it, but not to the extend of depleting its vigor, not in such a way as to induce a reaction upon the part of the host which will disrupt the desired association". In der Regel dürfte diese Harmonie fehlen und dürften beide Parteien versuchen einander zu schädigen, und sich gegen die Angriffe des anderen Partners zu verteidigen. Der ideale Zustand wäre natürlich ein Gleichgewicht zwischen beiden. Aber die Versuche, zu einem derartigen Gleichgewichtszustand zu gelangen, glücken nur in den seltensten Fällen. In der Regel schlägt der Wagebalken nach einer Seite aus. Jede der Parteien kann die Oberhand gewinnen. Durch dieses Wechselspiel beim Mobilisieren aller Kräfte bei beiden Parteien wird die Virulenz bedingt. Nur dann vermag man von Virulenz zu sprechen, wenn die biologischen Eigenschaften des Eindringers und des Wirtes gleichmässig berücksichtigt werden.

Zu den Factoren, welche die Pathogenität eines Microorganismus bedingen, gehören u.a. seine Invasionskraft, die Bildung von characteristischen Toxinen und bestimmten Enzymen, die Fähigkeit Hüllen zu bilden, um sich gegen die schädlichen Einflüsse der humoralen Abwehrkräfte des Wirtes zu verteidigen usw.

Zu den Factoren, welche die Empfänglichkeit und Resistenz eines Wirtes bedingen, gehören alle die bekannten Fähigkeiten der angeborenen natürlichen, nicht specifisch entstandenen Immunität, gehören die chemischen Stoffe, welche der Organismus herstellen kann, gehören die physikalischen Kräfte, die er mobilisieren kann, um der Infection Widerstand leisten zu können[1].

Gegen die Behauptung, dass viele Factoren nicht bekannt sind, wird wohl niemand Einspruch erheben können. Bei den Microorganismen kennen wir nur relativ wenige Factoren, durch welche ihre Pathogenität bedingt wird. In vielen Fällen haben wir keine richtige Vorstellung davon, wodurch nun ein bekannter Krankheitserreger seine krankheitserregende Kraft ausübt. Aber auch die Factoren der Empfänglichkeit und Resistenz sind noch viel zu wenig bekannt. So wissen wir erst seit verhältnismässig kurzer Zeit von der Bedeutung des Properdin-Systems für die Resistenz des Organismus gegenüber pathogenen Eindringern. Es ist durchaus denkbar, dass noch andere ähnliche oder ähnlich wirkende Systeme entdeckt werden.

Nur kurz sei erwähnt, dass auch die Untersuchung der Serumantistoffe mittels einer ähnlichen Technik sehr deutliche Resultate ergibt, auf die an anderer Stelle ausführlicher zurückgekommen wird.

Zusammenfassung.

Es wird eine Methode angegeben, um die Virulenz graphisch darzustellen. Der Verlauf der Curve wird durch Aenderung der Factoren sowohl des Erregers als auch des Wirtes deutlich beeinflusst.

Literatur.

Collier, W. A., de Roever-Bonnet, H. und Hoekstra, J. 1958. Trop. and Geog. Med. **10**, 261.

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[1] Anmerkung:

Nach Read (1958) ist Empfänglichkeit ein physiologischer Zustand des Wirtsorganismus, wobei der Parasit seine lebenswichtigen Stoffe geliefert bekommen kann. Unempfänglichkeit ist demgegenüber der Zustand des Wirtes, in dem lebenswichtigen Stoffe dem Parasiten nicht zur Verfügung stehen. Naturgemäss sind auch Zwischenstadien vorhanden.

Resistenz ist nach Read jene Veränderung in dem physiologischen Zustand des Wirtes, die eine Antwort auf frühere oder vorhandene Erfahrung mit dem Parasiten oder einer chemischen Einheit darstellt. Hierbei ist die Bildung von Antistoffen eingeschlossen (= specifische Resistenz).

Bei einem individuellen Wirt kann Empfänglichkeit und Resistenz gleichzeitig vorhanden sein. Ein hochempfänglicher Wirt kann auch hochresistent sein.

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ETUDE, AU CONTRASTE DE PHASE, DE L'ATTAQUE DE CERTAINS DESINFECTANTS VIS A VIS DES POLYEDRES DE *BORRELINA* VIRUS

par

M. S. JOLLY

(Reçu le 10 septembre 1958).

INTRODUCTION.

Dans la lutte contre les maladies infectieuses du Ver à soie, la désinfection est l'un des problèmes les plus importants. En effet, les conditions dans lesquelles une pièce d'élevage doit être désinfectée varient selon les régions, les climats et les types de bâtiments où sont réalisées les éducations. D'autre part, l'efficacité des désinfectants recommandés ne se confirme pas toujours dans la pratique. Ceci est particulièrement vrai en ce qui concerne les désinfectants utilisés contre le virus causant la plus importante maladie du Ver à soie: la Grasserie.

Souvent les désinfectants classiques employés contre les cryptogames et les bactéries ont été déclarés efficaces contre les virus. D'autres ont été testés empiriquement vis à vis de la Grasserie par évaluation de l'apparition de la virose après une infection globale précédant l'élevage test.

Ces techniques ont permis de reconnaître l'efficacité de certains désinfectants et ont facilité l'emploi rationnel de la désinfection en sériciculture. Elles n'ont cependant pas rendu possible la connaissance du mode d'action des désinfectants sur les virus et notamment les altérations que ces agents pathogènes ou leurs corps d'inclusion subissent au cours de la désinfection.

C'est pour cette raison que nous avons étudié les lésions que les corps d'inclusion de *Borrelina* virus subissent au contact de plusieurs produits en suivant les principes récemment définis par VAGO et ses collaborateurs (5, 6).

METHODES.

Nous nous sommes inspirés des travaux sur l'effet du suc gastrique vis à vis des corps d'inclusion (4,5) ainsi que des techniques mises au point à cet effet.

Après avoir déposé une goutte de suspension de polyèdres purifiés sur lamelles nous l'avons séchée à 30° C., durant 1/4 d'heure. Les désinfectants à tester à différentes concentrations ont été mis en contact avec les polyèdres secs par dépôt, d'une goutte prélevée dans les séries de dilution logarithmiques de chaque produit. La lamelle a été ensuite retournée sur lame creuse vaselinée et la préparation examinée au contraste de phase, grossissement 850. Les changements morphologiques survenus dans les polyèdres que nous examinions sans interruption ont été photographiés.

DESINFECTANTS TESTES.

Notre étude a porté sur les produits chimiques utilisés en sériciculture.

Ainsi, nous avons testé d'abord le formol, sous sa forme liquide, puis l'hydroxyde de sodium ou de potassium servant au lavage des murs et du matériel d'élevage (2). Le crésyl sous sa forme sodique et le phénol recommandés dans certains pays séricicoles ont également été éprouvés (3).

Enfin, nous avons tenu à connaître l'effet d'une substance peu utilisée en sériciculture, mais reconnue comme efficace en hygiène: le chlorure de chaux dont l'emploi est prévu en solution à 1%.

Les hypochlorites (sodium, potassium, et calcium) employés comme désinfectants des magnaneries ne figurent pas dans ces expériences car l'étude de ces produits a déjà été réalisée (5, 6).

Nous avons encore testé un désinfectant de moins en moins employé en sériciculture: le sulfate de cuivre, car son effet négatif vis à vis des viroses n'a pas été effectivement prouvé.

OBSERVATIONS.

a) Hydroxyde de sodium.

Cette substance employée à la concentration habituelle en sériciculture, soit 1%, a provoqué une désintégration immédiate ou très rapide des polyèdres. En préparant des séries de dilutions et de concentrations, nous avons pu suivre les modalités de cette dés-

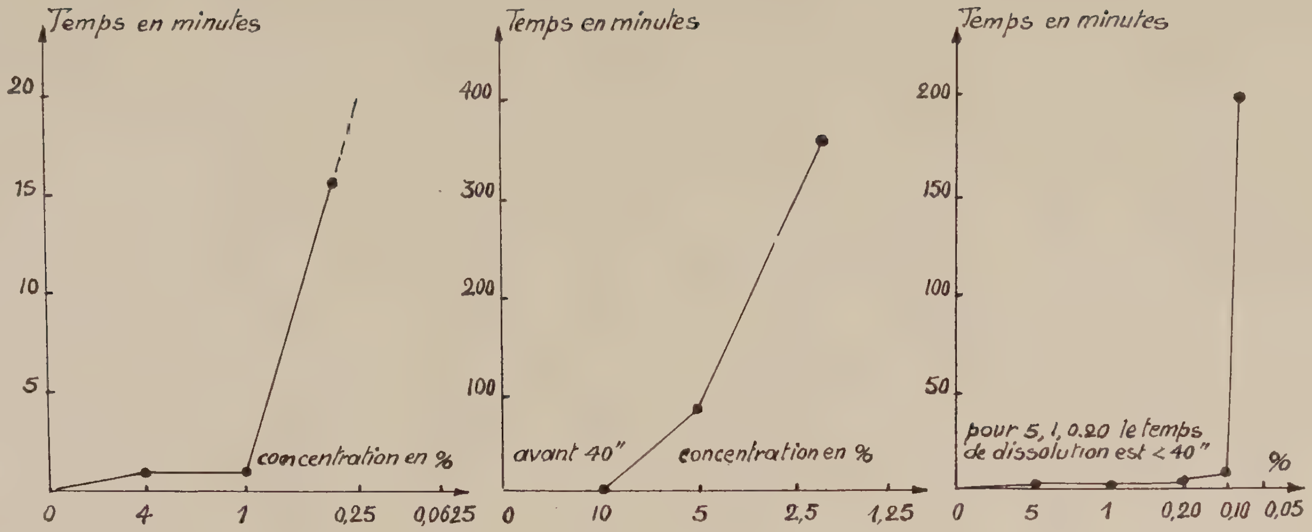

Fig. 1 Fig. 2 Fig. 3

Durée des délais pour destruction totale des polyèdres en fonction des désinfectants

Fig. 1. Hydroxyde de sodium. Fig. 2. Phénol. Fig. 3. Lait de chaux.

intégration. Sa durée est inversement proportionnelle à la concentration des solutions.

Ainsi, nous avons noté pour un groupe de polyèdres les temps suivants (fig. 1) : pour une concentration de 4% : moins de 40 secondes (temps minimum nécessaire pour effectuer la première observation) ; pour une concentration de 1% : moins de 40 secondes également ; pour une concentration de 0,25% : 16 minutes ; une dilution plus forte ne donne qu'une désagrégation partielle. Avec une solution de 0,0079125 %, les polyèdres ne sont pratiquement plus dissous.

En ce qui concerne les modalités de destruction, certains rapports existent également avec les concentrations. Les solutions fortes (4 et 2%) provoquent un éclatement des polyèdres dans un temps allant de 2 à 6 secondes.

Le produit plus dilué (1 à 0,25 %), permet de constater un processus débutant par la formation de fentes. Les parties ainsi isolées se détachent progressivement sous forme de fragments corticaux. Souvent sont libérés des corps centraux granuleux à contours circulaires. Bien que le détachement des fragments soit progressif, il se produit à certains moments une explosion. Les particules détachées subissent ensuite une dissolution totale (fig. 4).

Ce processus reste le même lorsque les concentrations sont encore plus faibles (0,1 à 0,01 %). Alors les fissures, le détachement des fragments corticaux et l'apparition des corps centraux se produisent également, mais ces fragments restent très longtemps ensemble et l'on voit aussi des polyèdres ou des lésions s'arrêter au stade "fissures".

b) Phénol.

A la dose indiquée pour la désinfection (2,5 %), les polyèdres se gonflent, puis se désagrègent assez lentement. En effet, une désagrégation rapide ne se produit qu'avec les solutions très concentrées (10 %) et elle devient nulle à partir de la concentration de 1,25 %. Les temps suivants de dissolution ont été notés (fig. 2) : à une concentration de 10 % : moins de 40 secondes (temps nécessaire pour effectuer les premières observations) ; à une concentration de 5 % : 95 minutes ; à une concentration de 2,5 % : 6 heures ; à une concentration de 1,25 % : aucune action.

Le phénol dilué à 10 % permet d'obtenir un changement dans la forme des polyèdres ; ils deviennent circulaires, se gonflent et se désintègrent rapidement. Leur région centrale se remplit de corps brillants (fig. 5). A la concentration de 5 %, le processus débute de la même façon mais la désintégration se produit irrégulièrement. Avec la solution à 2,5 %, la dissolution ne se termine pas et le processus s'arrête au stade du gonflement.

c) Crésyl.

Utilisé à 3 %, dose habituelle pour la désinfection des locaux d'élevage, il produit une action morphologique réduite. L'action est rapide avec les solutions très fortes et décroît en fonction de la concentration. Les temps de dissolution ne peuvent être notés car les polyèdres deviennent granuleux.

En ce qui concerne le mode d'action, la concentration à 50 % permet de noter divers stades. Tout d'abord, le polyèdre devient circulaire. Dans la région centrale on remarque de petits corps mais il ne sont pas brillants comme avec les solutions de phénol. Le polyèdre se gonfle énormément avant la désintégration qui commence par la membrane extérieure. Il se divise ensuite en parties incolores difficiles à délimiter (fig. 6).

Avec la solution à 12,5 %, nous avons noté le même processus mais plus lent. Toute dissolution est arrêtée à la concentration de 1,5625 %.

d) Chlorure de calcium.

Ce produit ne donne que de minimes traces de dissolution. A la concentration normale de 1,25 % aucune action. La concentration de 10 % donne une faible dissolution avec diminution de la diffraction de la lumière; le polyèdre devient gris foncé (fig. 7). Avec la solution à 5 % les polyèdres se fragmentent peu et certains ne sont même pas du tout influencés. Avec la solution à 2,5% la dissolution est arrêtée. Même après une heure de contact il n'y a que quelques polyèdres légèrement touchés.

e) Formol.

A la concentration habituellement utilisée pour la désinfection par mouillage (4 %) l'aldéhyde formique ne provoque aucun changement morphologique des polyèdres. Leurs contours restent intacts et la réfrigérence n'est pas diminuée. En augmentant la concentration ou en la diminuant, le comportement des polyèdres

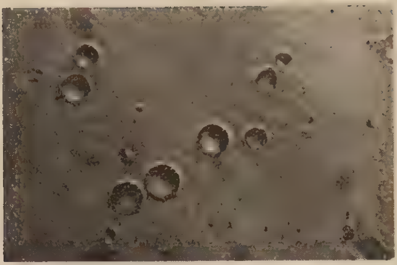

Fig. 4.

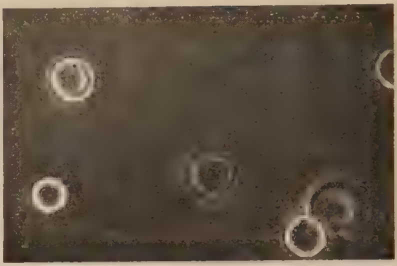

Fig. 5.

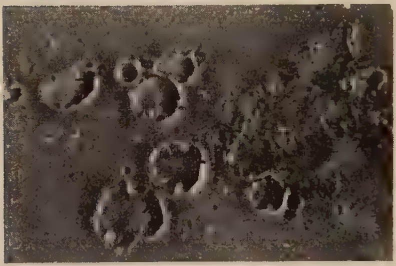

Fig. 6.

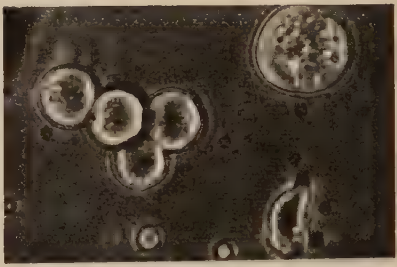

Fig. 7.

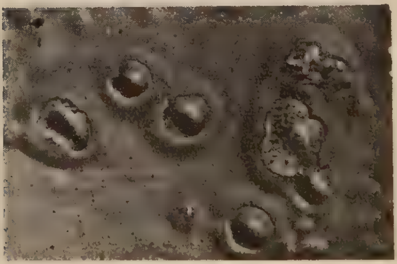

Fig. 8.

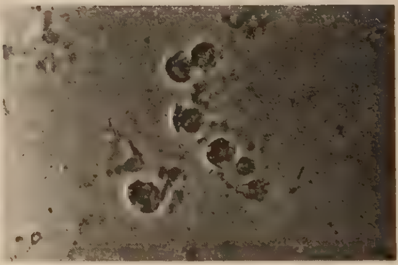

Fig. 9.

Divers caractères d'attaque des polyèdres en fonction des désinfectants. Contraste de phase 1 : 1000.

Fig. 4. Hydroxyde de sodium.
Fig. 5. Phénol.
Fig. 6. Crésyl.
Fig. 7. Chlorure de calcium.
Fig. 8. Formol.
Fig. 9. Lait de chaux.

reste le même. Il en est encore ainsi en prolongeant le temps nécessaire avec la concentration habituelle pour amener une inactivation des virus (fig. 8).

f) Sulfate de cuivre.

La solution à 10 % quelquefois employée pour la désinfection laisse les polyèdres intacts quelle que soit la durée de contact. L'augmentation de la concentration n'amène pas non plus de changement morphologique.

g) Lait de chaux.

Préparé selon le procédé habituel c'est-à-dire en utilisant le liquide surnageant de la chaux vive fraîchement éteinte, dans la proportion de 10 % les polyèdres sont nettement endommagés et dissous. Cet effet se produit d'une façon presque instantannée à 25 et 10 % et même à 5, 2,5 et 1 %. Ce n'est qu'à la concentration de 0,10 % que l'action est ralentie et les solutions plus diluée (0,05) n'ont qu'un effet insignifiant (fig. 3).

L'action du lait de chaux se traduit par un processus difficile à suivre à des concentrations fortes car, dès qu'il y a contact avec le produit, on constate la perte de brillance des polyèdres; ces corps disparaissent sans fragmentation. Par contre, à une concentration de 0,25 % on se rend bien compte des phases successives qui consistent en l'arrondissement des angles donnant une forme circulaire aux polyèdres et en l'apparition de petits fragments à l'intérieur. La conservation de ces fragments est assez longue avant que le polyèdre entier ne disparaisse progressivement (fig. 9).

Cette action peut être attribuée surtout aux composants hydroxyques qui accompagnent la formation du lait de chaux et qui ont une action alcaline.

Conclusions.

Ces observations montrent les effets morphologiques de plusieurs désinfectants sur les corps d'inclusion de la Polyédrie du Ver à soie. Elles revèlent les lésions variées que les polyèdres présentent en fonction de la nature des produits employés.

Certains de ces produits comme le formol ou le sulfate de cuivre ne déclenchent aucune modification morphologique, même pendant de longues périodes de contact. D'autres, surtout le groupe à effet alcalin direct, comme l'hydroxyde de sodium et le potassium, font éclater les polyèdres en fragments et dissolvent ceux-ci. Enfin, le phénol ou le crésyl modifient la forme des polyèdres en les gonflant quelquefois d'une façon particulièrement prononcée et en amenant une dissolution sans fragmentation.

Ces phénomènes doivent être interprétés en fonction de nos connaissances sur le pouvoir viricide de ces substances; connaissances mises à notre disposition par divers travaux récents en virologie des insectes. Il ressort des comparaisons effectuées qu'une action neutralisant le virus de la polyédrie n'est pas obligatoirement liée à une déformation morphologique des corps d'inclusion. Un exemple typique est donné par le formol inactivant les virus inclus dans les polyèdres avec une concentration ne provoquant aucune lésion. D'un autre côté l'inactivation des virus peut être liée à la désagrégation des polyèdres. Ainsi l'éclatement dû aux effets alcalins de NaOH, l'hypertrophie et la dissolution de corps d'inclusion dûs au phénol et au crésyl, sont accompagnés de la suppression du pouvoir infectieux.

En effet, le virus de la polyèdrie de *Bombyx mori* conserve sa virulence d'une façon exceptionnelle souvent pendant plus de 5 ans, grâce à la protection du corps protéinique que constitue le polyèdre et dans lequel il se trouve enfermé. Par contre, les virus libres dégagés des corps d'inclusion sont sensibles aux divers effets chimiques et climatiques (1). Ainsi la désagrégation des corps d'inclusion par les désinfectants est susceptible de transformer les éléments résistants en unités ayant une faible durée.

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(National Chemical Research Laboratory, South African Council for Scientific and Industrial Research, Pretoria, South Africa).

THE WINE YEASTS OF THE CAPE

PART II. — THE OCCURRENCE OF *BRETTANOMYCES INTERMEDIUS* AND *BRETTANOMYCES SCHANDERLII* IN SOUTH AFRICAN TABLE WINES[1]

by

J. P. VAN DER WALT and **AMELIA E. VAN KERKEN**

(Received January 17, 1959).

INTRODUCTION.

In Part I of this series VAN DER WALT and VAN KERKEN (1958) found that species of the genus *Brettanomyces* were the most prevalent yeasts in South African wines showing yeast hazes. The present communication deals with the taxonomy of *Brettanomyces* strains isolated in this study. In a detailed study of the genus *Brettanomyces* CUSTERS (1940) first proved that these yeasts, which had previously been encountered only in beers, also occurred in wines. He identified *Mycotorula intermedia*, a yeast isolated from French wine by KRUMBHOLZ and TAUSCHANOFF (1933) as a typical *Brettanomyces* species and considered it identical with *Brettanomyces bruxellensis*. BARRET, BIDAN and ANDRÉ (1955) similarly isolated strains of this genus from the yellow wines of the Château Chalon, but regarded them as a variety of the above mentioned species, *viz.*, *Brett. bruxellensis* var. *vini*. PEYNAUD and DOMERCQ (1956), on the other hand, compared the properties of several *Brettanomyces* strains, isolated from vinous products, with those of the type strains of *Brett. bruxellensis*, *Brett. lambicus* and *Brett. claussenii*, and considered the differences sufficient to establish two new species, *viz.*, *Brett. vini* and *Brett. schanderlii*.

These workers limited their study of the biochemical properties of the yeasts to the fermentation and assimilation of five sugars, namely, glucose, galactose, saccharose, maltose and lactose, and to the assimilation of nitrate. From this work *Brett. schanderlii* appeared to be a well-defined species, fermenting only glucose and galactose and not assimilating nitrate. The separation of *Brett. vini* as a new species was less clear cut. In view of this it was considered desirable, in the present work, to examine the fermentative and assimilative properties of the locally isolated strains as well as the type strains of the recognised *Brettanomyces* species on a more extensive basis.

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[1] Part I: Antonie van Leeuwenhoek **24**, 239, 1958.

MATERIAL AND METHODS.

Seventy-nine locally isolated strains were examined according to the standard methods employed by LODDER and KREGER-VAN RIJ (1952), WICKERHAM (1951) and CUSTERS (1940). Type strains of the following were also examined: *Brett. bruxellensis* (Kufferath et van Laer 1921), *Brett. bruxellensis* var. *non-membranaefaciens* (Kufferath et van Laer) Custers 1940, *Brett. lambicus* Kufferath et van Laer 1921, *Brett. anomalus* Custers 1940, *Brett. claussenii* Custers 1940, *Brett. schanderlii* Peynaud et Domercq 1956 and *Brett. vini* Peynaud et Domercq 1956.

In the assimilation tests the number of carbon compounds used was increased to thirty-two as suggested by WICKERHAM (1951) and the growth period extended to four weeks because of the slow growth of these organisms. The number of sugars in the fermentation tests was increased to ten, *viz.*, glucose, galactose, saccharose, maltose, trehalose, cellobiose, lactose, melibiose, raffinose, and melizitose.

RESULTS.

The results of the fermentation and assimilation tests for the type strains are recorded in Tables 1 and 2.

All seventy-nine of the locally isolated strains were characterised as *Brettanomyces* species by a) their somewhat slow growth; b) the accumulation of large amounts of acetic acid in aerobic cultures; c) a pronounced fermentative dissimilation; d) the absence of ascospore formation; and e) the formation of more or less "ogive" cells, and the production of a rather well-developed pseudomycelium, usually of the *Mycocandida* or *Mycotoruloides* type.

TABLE 1.

Fermentation reactions for the genus *Brettanomyces*.

	Glucose	Galactose	Saccharose	Maltose	Trehalose	Cellobiose	Lactose	Melibiose	Raffinose	Melizitose
Brettanomyces bruxellensis CBS 72	+	—	+	+	+	—	—	—	—	+
Brettanomyces bruxellensis CBS 74	+	—	+	+	+	—	—	—	—	+
Brettanomyces bruxellensis var. *non-membranaefaciens* CBS 78	+	—	+	+	+	—	—	—	—	+
Brettanomyces lambicus CBS 75	+	—	+	+	+	—	—	—	—	+
Brettanomyces anomalus CBS 77	+	+	—[1]	—	+	+	+	—	—	—
Brettanomyces claussenii CBS 76	+	+	+	+	+	+	+	—	+	+
Brettanomyces vini CBS 1943	+	+L	+	+	+	+	—	—	+W	+
Brettanomyces vini Bordeaux 48	+	+L	+	+	+	+	—	—	+W	+
Brettanomyces bruxellensis CBS 73	+	+L	+	+	+	+	—	—	+W	+
Brettanomyces schanderlii CBS 2796	+	+	—[1]	—[1]	—	+	—	—	—	—
Brettanomyces schanderlii Bordeaux CIII.	+	+	—[1]	—[1]	—	+	—	—	—	—

L = Latent fermentation, W = Weak fermentation.

[1] Slight fermentation may occur on prolonged cultivation in Durham tubes.

The formation of a pellicle was somewhat variable and not infrequently absent. The appearance of the streak cultures was variable.

On the basis of their fermentative and assimilative properties, the locally isolated strains fell into two distinct and well-defined groups.

Group I. Seventy-three strains fermented glucose, galactose (latently), saccharose, maltose, trehalose, cellobiose, melizitose and raffinose (weakly). Melibiose and lactose were not fermented. The following compounds were assimilated: glucose, galactose, saccharose, maltose, trehalose, cellobiose, raffinose, melizitose, D-ribose, salicin, α-methyl-D-glucoside, sorbitol (weakly), adonitol, glycerol, ethanol, potassium gluconate, succinic acid, malic acid and lactic acid. All strains utilised nitrate.

Group II. Six strains fermented glucose, galactose and cellobiose. On prolonged cultivation in the Durham tubes a slight fermentation of saccharose and maltose was sometimes observed. Other sugars tested were not fermented. The following compounds were assimilated: glucose, galactose, saccharose, maltose, cello-

TAI

Assimilation reactions fo

	Glucose	Galactose	Sorbose	Saccharose	Maltose	Trehalose	Cellobiose	Lactose	Melibiose	Raffinose	Melizitose
Brettanomyces bruxellensis CBS 72	+	—	—	+	+	+	—	—	—	+V	+
,, *bruxellensis* CBS 74	+	—	—	+	+	+	—	—	—	+	+
,, *bruxellensis* var. *non-membranaefaciens* CBS 78	+	—	—	+	+	+	—	—	—	+	+
,, *lambicus* CBS 75	+	+	—	+	+	+	—	—	—	+V	+
,, *anomalus* CBS 77	+	+	—	+L	+L	+	+	+	—	—	—
,, *claussenii* CBS 76	+	—	—	+	+	+	+	+	—	+	+
,, *vini* CBS 1943	+	+	—	+	+	+	+	—	—	+V	+
,, *vini* Bordeaux 48	+	+	—	+	+	+	+	—	—	+	+
,, *bruxellensis* CBS 73	+	+	—	+	+	+	+	—	—	+	+
,, *schanderlii* CBS 2796	+	+	—	+	+	+	+	—	—	+V	+
,, *schanderlii* Bordeaux CIII	+	+	—	+	+	+	+	—	—	+V	+

V = Variable assimilation, L = Latent assimilation.

biose, trehalose, D-ribose, salicin, α-methyl-D-glucoside (variable), sorbitol, glycerol, ethanol, potassium gluconate, succinic acid, malic acid and lactic acid. Neither nitrate nor nitrite was utilised.

Discussion.

In the present study the diagnosis of the genus *Brettanomyces* as defined by Custers (1940) and Lodder and Kreger-van Rij (1952) has been retained. As Wickerham (1952) pointed out the demarcation of this genus has been debated by various workers. Wickerham (1952) himself envisaged a phylogenetic line comprising both filamentous and non-filamentous, and nitrate positive as well as nitrate negative species for the genus. The isolation of *Brett. schanderlii* has certainly borne out Wickerham's views of the existence of the nitrate negative species.

Reviewing the assimilative and fermentative properties of the hitherto accepted *Brettanomyces* species, the following will be evident.

Brettanomyces claussenii, *Brett. anomalus* and *Brett. schanderlii* appear to be well-defined species showing a very clear differentiation from all other species of the genus. There appears to be a very close correspondence in the biochemical properties of *Brett. bruxellensis* strain CBS Nos. 72 and 74, its variety *non-membranaefaciens* CBS No. 78, and *Brett. lambicus* CBS No. 75. Moreover Lodder, Slooff

2.

genus *Brettanomyces*.

D-Arabinose	L-Arabinose	L-Xylose	Rhamnose	D-Ribose	Salicin	α-Methyl-D-glucoside	Mannitol	Sorbitol	i-Inositol	Dulcitol	Adonitol	Erythritol	Glycerol	Ethanol	Potassium gluconate	Succinic acid	Malic acid	Tartaric acid	Citric acid	Lactic acid	Nitrate
—	—	—	—	—	—	+	—	+	—	—	+	—	+	+	+	+	+	—	—	+	+
—	—	—	—	+V	—	+	—	+	—	—	+	—	+	+	+	+	+	—	—	+	+
—	—	—	—	—	—	+	—	+	—	—	+	—	+	+	+	+	+	—	—	+	+
—	—	—	—	+	—	+	—	+	—	—	+	—	+	+	+	+	+	—	—	+	+
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—	—	—	—	+V	+	+	—	+	—	—	+	—	+	+	+	+	+	—	—	+	+
—	—	—	—	+	+	+	—	+V	—	—	+	—	+	+	+	+	+	—	—	+	+
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—	—	—	—	+	+	+	—	+	—	—	+	—	+	+	+	+	+	—	—	+	+
—	—	—	—	+V	+	+	—	+	—	—	—	—	+	+	+	+	+	—	—	+	—
—	—	—	—	+	+	+	—	+	—	—	—	—	+	+	+	+	+	—	—	+	

and KREGER-VAN RIJ (1958) maintain that *Brett. lambicus* and *Brett. bruxellensis* differ only in the appearance of the cultures on malt agar.

In view of the above, the authors propose to unite these two species and the variety into a single species, *Brett. bruxellensis*.

In the case of *Brettanomyces bruxellensis* CBS No. 73 it is quite apparent that this strain is mislabelled since its fermentative and assimilative properties correspond in all respects with those of the two strains of *Brett. vini, viz.*, CBS No. 1943 and Bordeaux No 48. An inquiry into the origin of this mislabelled strain revealed that it was none other than *Mycotorula intermedia* which KRUMBHOLZ and TAUSCHANOFF (1933) had isolated from wine. On the basis of their unique fermentative and assimilative properties the maintenance of a separate species for this group of *Brettanomyces* strains isolated from wine is thus completely warranted, as PEYNAUD and DOMERCQ (1956) had claimed. The species epithet *vini* cannot, however, be maintained since the species epithet of KRUMBHOLZ and TAUSCHANOFF's organism has priority. In this connection, mention must be made of an organism which OSTERWALDER (1912) isolated from Swiss apple wine, *viz., Monilia vini*. This yeast was asporogenous, strongly fermentative and produced considerable amounts of volatile acid. The species formed both a well-developed pseudomycelium and true mycelium and may well have been a *Brettanomyces* species. Unfortunately, cultures of *Monilia vini* no longer exist, but as OSTERWALDER (1912) reported a fermentation of lactose for *Monilia* *vini*, it cannot be confused with either *Mycotorula intermedia* or *Brett. vini*. Accordingly, the correct nomenclature for the species in question is *Brettanomyces intermedius* Krumbholz et Tauschanoff 1933 (nov. comb.).

The fermentation and assimilation reactions of the locally isolated strains in Group I correspond very well with those of the three strains of *Brett. intermedius* (Tables 1 and 2). As these were isolated from no less than twenty-nine of the sixty wine samples examined by VAN DER WALT and VAN KERKEN (1958), it must be considered that *Brett. intermedius* is the main organism causing yeast hazes in South African wines.

The properties of the six strains of Group II agree very well with those of *Brett. schanderlii*. All six strains were isolated from one dry wine and were not found in any of the other fifty-nine samples examined. This species therefore appears to be less of a hazard in the South African wine industry than *Brett. intermedius*.

In connection with the ecology of the genus *Brettanomyces*, it is striking that these yeasts have so far exclusively been isolated from products of fermentation. Moreover, there appears to be a definite selection. While *Brett. intermedius* and *Brett. schanderlii* have only been isolated from wines or vinous products the other species have only been isolated from certain types of beers, *i.e.*, fermentation products of farinaceous origin. The restricted occurrence of these slow growing organisms in wines and beers may be due to two causes. The first is their alcohol tolerance which enables them to compete successfully with faster growing but less alcohol tolerant yeasts. The second may be their growth factor requirements. In an examination of the vitamin requirements of the type strains of the species, it was found that all required biotin and thiamine. In view of this, and the observation by WICKERHAM (1952) that moribund *Brettanomyces* cells could be induced to active growth by the presence of certain other actively growing yeasts, it seems most likely that *Brettanomyces* species may depend for their growth factors on the activities of auxo-autotrophic yeasts used in fermentation. In addition, the raw materials employed in the manufacture of wine or beer, *i.e.*, grape must or beer wort, are in themselves relatively rich in growth factors. Nevertheless, the genus *Brettanomyces* may have a wider distribution than hitherto reported. Their apparently limited distribution is most probably due to the sharp competition of the more robust and more rapidly growing species.

Summary.

The biochemical and morphological properties of seventy-nine strains of *Brettanomyces* isolated locally from South African wines together with those of the type strains of *Brett. bruxellensis*, *Brett. bruxellensis* var. *non-membranaefaciens*, *Brett. lambicus*, *Brett. anomalus*, *Brett. claussenii*, *Brett. vini* and *Brett. schanderlii* were studied.

It was established that whereas *Brett. anomalus*, *Brett. claussenii* and *Brett. schanderlii* are well-defined species, *Brett. bruxellensis*, its variety *non-membranaefaciens* and *Brett. lambicus* constitute a single species. The authors propose to unite the two species and variety into a single species *Brett. bruxellensis*.

From the study of the type strains it appeared that *Mycotorula intermedia* and *Brett. vini* are identical and that the correct nomenclature for the species in question is *Brettanomyces intermedius* nov. comb.

Seventy-three strains locally isolated from twenty-nine samples of wine were found to be representatives of the species *Brett. intermedius*. The six remaining strains, isolated from one wine sample, were found to correspond to *Brett. schanderlii*.

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THE APPLICABILITY OF EMMERLING'S PRINCIPLE ("MOULD TEST") IN FOOD MICROBIOLOGY

by

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(Received March 27, 1959).

In 1890 Emmerling described a test for the evaluation of the microbiological quality of feeds, which consisted of moistening the product under investigation and then incubating it for one, or a few, days at about 35 °C. Upon the completion of this test a feed of good quality was supposed neither to have become mouldy nor to have been decomposed by bacterial action (3). Obviously, this test is a simple sterility test, although it has generally been called a "mould test".

From the beginning this procedure was severely criticized (17, 18, 7, 19, 9, 5) and, accordingly, did not gain any general acceptance in food microbiology (6). However, because of its simplicity, it was nevertheless adopted in 1924 for the microbiological evaluation of cereal products, cocoa and spices, in Royal Decrees under the Netherlands Food Law. Ever since, the test itself as well as its significance has been repeatedly studied, discussed and criticized by Dutch microbiologists (6, 16, 10, 2, 1, 12). From these discussions it appeared, *inter alia*, that the test had been introduced in food inspection in The Netherlands, in order to procure information on one or more of the following microbiological properties of foods:

(a) Stability during future storage, *i.e.* "shelf life";
(b) The occurrence in the food of microbial proliferation short of the stage of organoleptic detectability;

(c) The microbiological quality of the raw materials from which the product under investigation had been prepared;
(d) The conditions of preparation and storage of the product.

From these considerations it becomes obvious at once why Emmerling's mould test has been very little applied in food bacteriology.

(i) Regarding the prognosis of keeping quality, it is clear that a test of the type proposed by Emmerling is of no value; because the stability of dry foods such as cereals, cocoa and spices depends entirely on the equilibrium relative humidity under which the food is stored, and not on its microbiological status (13).

(ii) It can be understood *a priori*, and it has indeed been shown repeatedly by experiment, that foods of excellent bacteriological quality and containing only a few mould spores per gramme may give a very unfavourable mould test, because the moistened food under investigation is an outstanding medium for mould growth (18, 5, 6, 2); while partly deteriorated products fairly often show a negative Emmerling test (17, 9, 5, 10), as a consequence of less favourable conditions in the moistened commodity.

Therefore it is not possible, in general, to decide from the result of an Emmerling test whether a food has been prepared from raw materials of good microbiological quality, handled in a sanitary way and stored *secundum artem*, nor whether it has been subjected to microbial proliferation or even slight deterioration. However, in a few specific cases *e.g.* in deciding whether re-infection has occurred after the regular heat treatment, in products such as cocoa, rolled oats and partially precooked rice, the test can be of incontestable value for plant control work (*vide infra*).

(iii) For any of the purposes mentioned under b, c and d above, reliable and more specific methods have been worked out, before or since Emmerling's test was published.

The required information regarding c and d can be obtained by selective cultural techniques and microscopic methods. Whether a food has been subjected to microbial deterioration to a degree which may soon lead to abnormal taste, odour or consistency, can be ascertained in the same way and also, in a few cases, by simple chemical tests such as determination of the acid number, ammonia content, etc.

Yet, Emmerling's test has always maintained its charm for food plant bacteriologists and for food inspection purposes, because of its incontestable simplicity, and because results are known within 24 hours. Therefore, food inspection laboratories in The Netherlands have been reluctant to eliminate it from their standardized methods, while one of us (8) recently indicated its usefulness in industry for the specific cases just mentioned under (ii). Therefore the purpose of this paper will be to consider where Emmerling's original test or suitable modifications can be applied, and where its use is to be definitely discouraged.

Use of Emmerling's test in food mycology.

Principles of a more suitable test.

It goes without saying that, in principle, the presence of fungal spores in foods can be detected with the help of Emmerling's test. For this purpose the following conditions must however be fulfilled:

(i) The equilibrium relative humidity (e.r.h.) of the moistened product, as subjected to the test, must be such as to permit rather prompt spore germination and subsequent mycelial growth; *i.e.* the e.r.h. must be of the order of 0.85 or higher (13).

(ii) The temperature of incubation of the moistened commodity must preferably be optimal for moulds: *i.e.* in the range 20—25° C., and not about 35° C., as originally suggested by Emmerling and maintained ever since, despite repeated theoretical objections (10, 2, 12) and although Emmerling himself (4) accepted the desirability of incubating at lower temperatures as early as 1899.

(iii) Incubation must be continued for at least three days, in order to enable all mould spores, which are generally much slower in their development than bacteria (13), to germinate and form mycelia.

(iv) The activity of bacteria or bacterial spores must be precluded in the test, so that the growth of any moulds in the products may not be inhibited by bacterial antagonism.

For this reason, one of us (11) has suggested limitation of the e.r.h. in the test to a value of 0.90 which permits rather prompt fungal growth, but inhibits the development of bacteria. Because this procedure involves the use of specially equipped incubators, it seems preferable for routine purposes to inhibit bacterial growth by adding antibacterial antibiotics, such as penicillin (7500 U/l) + streptomycin (10 mg/l) [1]), or a tetracyclin antibiotic at a level of 100 μg/ml of water used for moistening the product.

(v) The test can only be applied to foods which do not contain antimicrobial agents in effective concentrations.

For example, cloves and cinnamon are among the products which cannot be tested in this way, unless the antimicrobial essential oils have been extracted prior to the test; a procedure which renders the test rather complicated and so deprives it of any essential advantage over current methods for the microbiological analysis of foods.

(vi) Also, the EMMERLING type of test cannot be applied to foods such as potato starch or maizena, which do not contain enough nutrilites to permit the development of microbes (13) unless appropriate nutrients are added, *e.g.* in the form of 0.25 % of dehydrated yeast extract.

Limitations of the modified test.

It is our experience that the EMMERLING test, modified according to the principles outlined above, can be used for the qualitative detection of postprocessing contamination with moulds of such foods as cocoa and rolled oats.

It also gives reliable results when used for the semi-quantitative enumeration of moulds in some foods. However, the quantitative figures obtained in this test (*viz.*, the numbers of mould colonies developing from 1.5—2.0 gramme of commodity) may sometimes be erroneously low, and are always unreliable if more than the order of 30 mould colonies develop per plate. We have the impression that the occasional low counts are often caused by *Rhizopus* species, which tend to outgrow moulds which do not form so copious an aerial mycelium (10) and moreover obscure the few colonies of the latter type which do develop.

Procedure.

The recommended procedure for the use of EMMERLING's principle in food mycology is as follows.

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[1]) This formula was kindly suggested to the authors by Dr L. E. DEN DOOREN DE JONG, Coolsingel Hospital, Rotterdam, The Netherlands.

A quantity of 1.5—2.0 grammes of the food is weighed into sterile petri dishes. This is then homogeneously moistened with 2 ml of sterile tap water (in the case of potato or corn starch, with a sterile 0.5% solution of dehydrated yeast extract), and with 1 ml of a solution of 50 mg oxytetracyclin ("terramycin") or chlortetracyclin ("aureomycin") in 500 ml sterile distilled water. If the latter solution is stored, it should be kept at about 5° C. for not longer than 1 month or otherwise at —20° C.

The plates are incubated at 20—25° C. and inspected after 1, 2, 3 and 5 days.

Results.

Table 1 gives a selection of the results of tests carried out according to this procedure.

In order to show, once more (10, 2, 12), the inadequacy of the Emmerling test with incubation for 24 hours (or more) at 37° C. as originally recommended, there are also given the data obtained when applying the latter procedure to the same samples. It is obvious that false negatives or extremely low "counts" are generally obtained with the incubation temperature of the original Emmerling test.

As has been said, the data in Table 1 give only a selection of figures, *viz.*, those which permit a quantitative comparison. While

TABLE 1.

A comparison of a selection of results of the classical Emmerling "mould test" and of the modified assay, as applied to cereals and cocoa.

Commodity	Numbers of mould colonies per 1.5 gramme			
	Count in glucose/ yeast extract/agar of pH = 3.5 (5 d/25 °C.)	Classical test, 37 °C. Incubation (days)		Modified assay (> 3d/25 °C.)
		1	3	
Cocoa SN 1	1.0×10^2	0	0.06×10^2	1.0×10^2
SN 2	1.5×10^2	0	0.07×10^2	1.1×10^2
SN 3	0.7×10^2	0	0.08×10^2	0.7×10^2
SN 4	1.0×10^2	0	0.03×10^2	0.3×10^2
SN 5	0.2×10^2	0	0.02×10^2	0.2×10^2
SN 6	1.2×10^2	0	0.01×10^2	0.4×10^2
Rolled oats, SN 1	0.5×10^2	0	0.01×10^2	0.2×10^2
SN 2	0.5×10^2	0	0	0.2×10^2
Rye flour	0.4×10^3	0	0	0.08×10^3
Wheat flour	0.6×10^3	0	0.01×10^3	$>0.01 \times 10^3$*

*) Exact counting virtually impossible due to preponderance of aerial mycelium.

the results recorded for cocoa and rolled oats represent the average of the vast amount of data collected, the figures for the other cereals are the most favourable ones, *i.e.* the very few that permitted more or less exact counting of the moulds developed in the Emmerling test, carried out in the presence of aureomycin and at 25 °C. From these facts it follows that even the modified Emmerling test, although it can be recommended for testing cocoa and rolled oats, can only to a much lesser extent be used for the mycological analysis of other cereals.

Use of Emmerling's test in food bacteriology.

If, for reasons of simplicity, it is explicitly desired to avoid plate counts, Emmerling's principle can also be modified so as to permit the growth of bacteria only, which one can then attempt to count. For this purpose we found effective the application of the new, and very potent, completely specific antifungal antibiotic pimaricin (20). Pimaricin was kindly made available to us by the manufacturers, the Royal Dutch Yeast and Fermentation Industries, Delft, The Netherlands. When an aqueous solution of 25 μg/ml pimaricin is used for moistening the food, virtually no development of yeasts or moulds is observed, and the only organisms manifesting their presence are bacteria.

Here, even more than in the mould test, application of the Emmerling principle requires products of very low infection. We found that, if more bacteria than the order of 10 per gramme of food are present it is virtually impossible to count them even approximately; while this can be done, be it with some difficulty, when there are only few of them, in which case their "colonies" can be recognized as wet or coloured spots. Hence for products containing more than very low numbers of bacteria, *e.g.* spices and most unheated cereal flours, the procedure loses all significance. The test is therefore to be considered as a kind of sterility test — just as is the mycological test. It has, however, even for this purpose, a rather serious shortcoming in that obligate anaerobic bacteria cannot be detected in this way.

Also, being a sterility test, all aseptic precautions must be taken here which are normally observed in sterility testing, to prevent bacteria introduced into the plates during moistening etc. from being mistaken for organisms originally present in the food (14).

Hence it is highly questionable whether, if only for practical reasons, it would not be much more recommendable to carry out ordinary bacterial plate counts rather than adhere to EMMERLING's principle as a test for bacteria in foods.

PURPOSES FOR WHICH THE EMMERLING TEST SHOULD NOT BE USED.

While the benefits of the use of EMMERLING's principle in food bacteriology in general may be debatable, it is certain that a test of this type is valueless for verifying the wholesomeness of foods, and should never be contemplated for that purpose. Yet, clinging to the EMMERLING type test because of its simplicity and rapidity involves the risk that it will be used as the only criterion for the microbiological status, *e.g.* of precooked dry foods, which are to be consumed without any prior heating. Among these are precooked cereal infant foods, instant pudding, cold swelling rolled oats, puffed rice, cereal flakes, soluble cocoa and mixtures of cocoa, sugar and milk powder.

Contaminated products of this type present a great health risk for the, mostly juvenile, consumers; especially if the reconstituted products are stored for a considerable period of time without refrigeration. Therefore, a non-specific test, such as that of the EMMERLING type, is far from sufficient here — although as has been said, it may help in judging the general microbiological quality ("sterility") of the products and so be of value in plant control.

What is required for the final evaluation of the bacteriological quality of such instant foods is a series of specific and quantitative tests for pathogenic bacteria, and for the appropriate indicators of faecal or other contamination of foods. The specific requirements for these products have been studied and are reported elsewhere (15).

Summary.

EMMERLING's test, wherein feed is moistened and then stored at elevated temperatures and inspected for mould growth ("mould test"), has been adopted for the microbiological evaluation of cereal products, cocoa and spices in Regulations under The Netherlands Food Law. In spite of repeated criticism both in Germany and in The Netherlands, the procedure has hitherto been

maintained because of its incontestable simplicity and because results are known within 24 hours.

It is shown that the test can in fact be used, in principle, for the mycological evaluation of products, which are heated in the course of their industrial preparation, such as cocoa and rolled oats and to a lesser extent also of other cereals and spices which do not contain antimicrobial substances. It is prerequisite here, however, that (i) simultaneous bacterial development is inhibited by the addition of chlortetracyclin or oxytetracyclin; (ii) the temperature of incubation is 20—25 °C.; (iii) incubation is continued for at least three days.

It is demonstrated that the usefulness of a procedure of the type of the EMMERLING mould test in the bacteriological examination of foods, *i.e.* a test wherein simultaneous development of moulds and yeasts is inhibited by the addition of the antibiotic pimaricin, seems very doubtful, save perhaps for presumptive laboratory control in factories preparing preheated dry foods, which are virtually sterile.

It is explained that a non-specific test of this sort should definitely not be considered for final Public Health control, *e.g.* of precooked cereal foods, but that the usual quantitative tests for pathogenic bacteria and for indicator organisms are imperative here.

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ARE SPIROCHAETES BACTERIA OR PROTOZOA?

by

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(Received September 30, 1958).

In my book (VAN THIEL, 1948) I expressed as my opinion that spirochaetes have more affinities with protozoa than with bacteria. I mentioned seven features indicating this relationship, partly after ZUELZER (1931). These features are:

1. In *Leptospira* no distinct ectoplasmatic envelop is present.

2. Division takes place by transverse fission, differently in spirochaetes (*viz.*, by stretching and thinning of the protoplasm and then its breaking) than in bacteria, where a transverse wall is formed.

3. Most species of spirochaetes are, like protozoa, soluble in lipoid solvents (best in 5% bile salts and hemolytic substances); in bacteria this is seldom the case.

4. The appearance of intermediate hosts in a few spirochaetoses, *viz.*, febris recurrens and spirochaetosis duttoni. In as far as lice and ticks cannot infect man until a certain length of time has elapsed after the infective feed with species of *Borrelia*, they are comparable with mosquitoes as intermediate hosts of malaria parasites, glossina of trypanosomes and ticks of piroplasms.

> Although there is some sort of development no true development can be assumed, since the view held by LEISHMAN, HATT and NICOLLE (quoted by BURGDORFER, 1951) has been abandoned.

The spreading of infection of plague bacteria by fleas, Salmonellae by the bug *Triatoma* (LIEM SOEI DJONG, 1958) or Rickettsiae by the louse occurs by quite other means.

5. The regular occurrence of relapses, to be found in a few spirochaetoses (notably febris recurrens and in a lesser degree in Weil's disease and lues), makes one think of malaria. Bacterial

diseases may relapse only in a few patients, as is the case in typhoid fever.

6. With the exception of the leptospiroses, spirochaetoses are curable by mercury and by arsenic compounds, which is also the case in trypanosomiasis and in malaria.

Dr Gordon Davis called my attention to his view, as expressed in ,,Review of the Spirochaetes'' (1948), that the preponderance of evidence would indicate that the arsenicals have been highly overrated for the treatment of the relapsing fevers. This would then restrict the arsenicals only to the trepanomatoses. Mercury and arsenic compounds, however, are not specific for the treatment of malaria. So the results of treatment appear to furnish but a weak argument in favour of relationship with the protozoa, all the more so as rat bite fever is a bacterial disease and is cured by salversan.

7. A serological property: the lytic processes in immune sera of man and animal infected by *Leptospira* take place without the cooperation of complement, which is the opposite to what happens with bacteria. In serological tests, lysins as well as agglutinins play a part in the reaction, and this occurs with active and inactivated serum.

Relationship with spirilla among the bacteria may be claimed by:

1. Presence of a delicate cell wall in *Treponema* and *Borrelia*.
2. The transverse fission.
3. Absence of polar differentiation.
4. Absence of a morphologically differentiated nucleus.

Considering the above arguments I concluded (1948) as to a greater relationship of spirochaetes with protozoa, although by then no evidence of an axial filament had been found and Mudd, Polevitsky and Anderson (1943) had found relatively long lophotrichous flagella on different places of the cell body of *Treponema pallidum* (followed in 1948 by Hampp, Scott and Wyckoff for the cell body of *Borrelia vincenti*). I explained these flagella as artificial structures caused by contact with distilled water. Nor had Morton and Anderson (1943) found such flagella.

,,Buds'' and ,,granules spirochétogènes'' were not involved in my view of the relationship, because of our insufficient knowledge of these structures. The same applies for ,,spherical bodies'' or ,,microcysts'' in *Spirillum* (Williams and Rittenberg, 1957).

Since 1948 more has become known about some of the above mentioned structures.

Cell wall.

I had stated that no distinct ectoplasmatic envelop is present in *Leptospira biflexa.* This interpretation of my electron-micrograph has appeared incorrect, as may be learned from electron-micrographs published later. BABUDIERI (1948), BRADFIELD and CATER (1952), CZEKALOWSKY and EAVES (1955) found in *Leptospira* an extremely thin and delicate cell membrane. As a result of better preservation of the essential structures of *Leptospira* a cell wall is defined with great clearness in the electron-micrograph of *Leptospira canicola* by SWAIN (1957). This delicate cell wall, however, does not argue against the relationship with protozoa. I refer to the electron-micrographs by ANDERSON, SAXE and BEAMS (1956) of *Trypanosoma equiperdum* and those by LUDVIK (1958) of *Toxoplasma gondii,* where the outline of the cell is much more distinct. The latter parasite is certainly a protozoan (VAN THIEL, 1956). The cell wall of bacteria is much thicker.

Filaments.

These are surely extant in spirochaetes. BABUDIERI showed for the first time an axial filament in *Leptospira* and he drew my attention to the visibility of this filament in my electron-micrograph 1 of *Leptospira biflexa* (1948). I also found a distinct axial filament in an, at that time, disapproved photo (VAN THIEL, 1958).

The presence of one axial filament in *Leptospira* has also been observed by BRADFIELD and CATER, CZEKALOWSKY and EAVES, and SWAIN (1955, 1957). A bundle of fibrils occurs in *Treponema* and *Borrelia.* In *Treponema* there are 3 fibrils in the bundle (SWAIN, 1955), in *Borrelia duttoni* about 8 (BRADFIELD and CATER), 8 to 12 (SWAIN, 1955) and in *Borrelia recurrentis* 15 to 20 (BRADFIELD and CATER), 8 to 12 (SWAIN 1955).

Taking into account the electron-micrographs of *Leptospira* by FULTON and SPOONER (in ALSTON and BROOM, 1958) there is no longer reason to doubt whether the axial filaments and the cytoplasmic element are wound round each other.

Less unanimity exists concerning the position of these fibrils with regard to the cell wall and I think it best to leave the question whether the axostyle in *Leptospira* is in or outside the cell wall undecided. According to BABUDIERI and to SWAIN (1955, 1957) a straight axial filament with the spiral of protoplasm twisted around it within the cell membrane is found in *Leptospira*; according to BRADFIELD and CATER and to CZEKALOWSKY and EAVES this fibril lies outside the membrane. Concerning the situation inside the cell wall I agree with SWAIN on the evidence of the slight conspicuousness and non-protrusive outline in the intact *Leptospira* and of the fact that, when the cell wall is removed by disintegration or by bile salts, the filament becomes more clearly defined and distinct. On the other hand, neither does SWAIN's picture number 3 prove that the axostyle is situated within the cell wall, nor does the electron-micrograph made by CZEKALOWSKY and EAVES show clearly the presence of grooves on the protoplasmic cylinder, where the axostyle formerly laid in leptospires treated for a short period with a low concentration of sodium desoxycholate.

According to SWAIN (1955) in *Treponema pallidum* an axial band of 3 fibrils, intimately applied to the coiled cytoplasm of the cell and placed within the cell membrane, is to be found.

In *Borrelia duttoni* and *Borrelia recurrentis* the bundles of fibrils lie inside the cell membrane according to BRADFIELD and CATER, but SWAIN (1955) found them to lie superficially, covered only by a slime layer and wound around the spirochaete on the outerside of the cell membrane. In contradistinction the fibrils of *Borrelia vincenti* are disposed within a cell membrane.

It may be concluded that one axial filament exists in *Leptospira* and an axial band of 3 fibrils in *Treponema*, both probably situated within a cell membrane. In *Borrelia* the bundle of fibrils may be either within the cell membrane or outside it.

At present it is uncertain whether the fibrils are contractile (SWAIN, 1955) or merely serve as stiffeners (SWAIN, 1957), or that they serve as a means of locomotion, though in that case they need not be contractile (CZEKALOWSKY and EAVES). According to BRADFIELD and CATER the balance of evidence is in favour of their being contractile. In an electron-micrograph by FULTON and SPOONER (in ALSTON and BROOM, 1958) cross-striation is visible on one axial filament, a fact which points to contractibility. The locomotion of spirochaetes can probably best be explained by assuming that the fibrils have at the same time a ,,skeleton'' function in the rigid *Leptospira* and *Treponema*. This view is also held by CZEKALOWSKY and EAVES.

BRADFIELD and CATER rightly assert that the existence of the system of fibrils seems to be a unique structural device found in all the spirochaetes, but only in these organisms. They may serve as a means to define the group as a whole, and to distinguish it clearly from all related micro-organisms. According to SWAIN (1955) the presence of fibrils suggests a degree of organization intermediate between bacteria and protozoa. As far as I know no internal and external fibrils which do not function as flagella are found in bacteria. In protozoa on the other hand delicate intracellular fibrils exist in *Toxoplasma* (BRINGMANN and HOLZ, 1954; VAN THIEL, 1956; LUDVIK, 1958).

BABUDIERI and BOCCIARELLI (1948) and WATSON *et al.* (1951) remarked on the striking resemblance of the bundles of fine filaments twisted around the bodies of spirochaetes to the undulating membrane of some protozoa. BRADFIELD and CATER studied *Cristispira balbiani* from the intestine of the oyster. The veillike crista or crest is a particularly thick, spirally wound bundle of some hundred or more fibrils - so thick that it is apt to splay out into something very much like the undulating membrane of a trypanosome, with the result that the organism was first named *Trypanosoma balbiani*. The resemblance is, however, quite spurious. The fibrils tend to remain closely wrapped around the organism outside the cell membrane during life, forming only a low crest and only after death do the fibrils splay out into the broad membranous crista shown in most published accounts. The electron-micrographs of trypanosomes made by KLEINSCHMIDT and KINDER (1950), by KRANEVELD, HOUWINK and KEIDEL (1951) and by ANDERSON, SAXE and BEAMS (1956) do not indicate any resemblance of the bundle of filaments in spirochaetes to the undulating membrane of trypanosomes. Rather do the evenly spread, nearly parallel, cork-screw-shaped fibrils, which may be assumed to form part of the cell membrane of trypanosomes and which fit in perfectly with the spiral twisting of the trypanosome body (KRANEVELD, HOUWINK and KEIDEL), point to a relationship with the more compact bundle of fibrils in spirochaetes.

Flagella.

The micrographs which made MUDD *et al.* (1943), MUDD and ANDERSON (1944), MORTON *et al.* (1951) suggest that *Treponema pallidum* was flagellated, were of strains prepared by washing in distilled water, without preliminary osmic-acid fixation. These spirochaetes for the most part lack the constant regular spiral morphology of *Treponema pallidum* and they may represent damaged or effete organisms whose shape has been distorted by the severing of internal fibrils which were then displaced to resemble bacterial flagella (Swain, 1955). When fibrils are broken by peptic digestion, similar appearances will result (Swain, 1955). The presence of flagella therefore can no longer be used as an argument for a relationship with bacteria, neither does it fit in with the movements of spirochaetes.

Williams and Rittenberg (1957) published taxonomic studies of the genus *Spirillum* Ehrenberg (1832). It belongs to the *Eubacteriales* being characterized by the presence of bipolar tufts of flagella in most species, by the mode of cell division and by the histochemical criteria considered critical for the demonstration of chromatinic bodies in bacteria.

They record as reasons for the relationship of spirochaetes and spirilla:

1. The striking similarity in the postulated nature (concerning contractility) of the fibril bundles of spirochaetes and the structure of the bacterial flagella. No compelling reason would exist to believe in an origin of the spirochaetes independent of the *Eubacteriales*. Very little evidence would justify therefore a wide separation of the spirilla and the spirochaetes.

2. The flexuousness of the body, *viz.*, the ability to bend or flex, the formation of secondary waves that are often imposed on the primary coil of the pathogenic spirochaetes. Not only do the spirilla bend or flex, but they also straighten during motion and increase in length as was shown by Pijper *et al.* (1953).

3. The presence of a cell wall. The spirilla and spirochaetes differ only quantitatively on this point.

None of these three reasons can be accepted by the present author, for the following reasons:

Ad. 1. A supposed identical function of fibril or fibril bundle in spirochaetes and of flagellum in bacteria is not an argument for a genetical relationship. With equal justification a relationship could be postulated for fibrils of spirochaetes with flagella of flagellates.

Ad. 2. The ability of spirilla to bend or flex belongs to spirilla as well as to protozoa.

Ad 3. The very thin wall of spirochaetes does not point to a relationship of spirochaetes with bacteria (see above).

Summarizing I answer to the question whether spirochaetes are bacteria or protozoa: neither, because I imagine the origin of spirochaetes from a hypothetic archetype of life, next to bacteria and protozoa.

Their features may therefore resemble more or less those of bacteria and those of protozoa. The above enunciation may have made clear that I hold the view that most features of spirochaetes show closer relations to protozoa than to bacteria.

From this point of view it would seem a logical conclusion to classify spirochaetes within the *Protozoa*, as follows:

subregnum *Protozoa*
class *Spirochaetoidea* Dobell, 1912
family *Spirochaetidae* Swellengrebel, 1907
genus *Spirochaeta* (type species *S. plicatilis*)
  subgenus 1. *Cristispira* (type species *C. balbiani*)
  subgenus 2. *Leptospira* (type species *L. biflexa*)
  subgenus 3. *Treponema* (type species *T. pallidum*)
  subgenus 4. *Borrelia* (type species *B. recurrentis*)

I can, however, accept that practical reasons make it desirable that spirochaetes remain included in the subregnum *Bacteria* (according to Breed, Murrey and Smith, 1957) : *a.* by following Alston and Broom (1958) in reasoning that zoological differentiation depends on morphology and that there are no morphological differences which could be used to subdivide the genus *Leptospira*. This seems to be the case for *Treponema* as well, probably not for *Borrelia*. *b.* By my point of view that spirochaetes are a ,,transition group'' on the borderline of Protozoa and Bacteria.

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THE DETECTION OF FAECAL STREPTOCOCCI IN DRINKING-WATER

by

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The method originally prescribed for the detection of (faecal) streptococci in drinking-water was very simple. Louwe Kooijmans (1930) as well as Meerburg and Massink (1933) state that the water sample one wishes to examine is added to a glucose broth. If after 48 hours' incubation at 37° C. a sediment has formed, a Gram stained preparation is made of this.

A simple preparation stained with gentian violet is also allowed. A microscopic examination is then made for the presence of streptococci (streptococci isolated from water appear in the form of diplococci or in very short chains). Obviously the idea here is, that all the organisms isolated in this way are of faecal origin.

In practice, however, this simple test was sometimes found to present difficulties, so that a further identification proved desirable (Spaander, 1941). This led on the one hand to the development of methods in which the glucose broth was enriched with substances that check the growth of other organisms without affecting the streptococci. Thus caffeine (Folpmers, 1939), sodium azide (Mallmann and Seligmann, 1950) and thallium sulphate (Dresscher, 1955), for instance, were added; the first-named substance is no longer used.

On the other hand solid media began to be used, chiefly blood-agar and agar containing potassium tellurite. This made it possible to cultivate pure colonies whose characteristics can be further investigated.

The English instructions (Ministry of Health, 1956. Report no. 71. The bacteriological examination of water supplies, p. 34, London), provide an example of this. In the so-called "direct azide method" the water sample is added to the medium of Hannay and Norton, which contains azide, and incubated at 45° C. If acid is formed whithin 18 hours, which is shown by the change of colour of the added brom-cresol-purple, the presence of streptococci may be taken for granted. If no acid has been formed after 18 hours, incubation is continued until 48 hours in all have elapsed. If acid has been formed now, the presence of streptococci must be further confirmed. This is done by growing a pure culture on McConkey agar or on mannitol-bile-salt neutral-red agar. Small, acid-forming (i.e.red) colonies indicate the presence of streptococci. If required, further proof of the presence of *Str. faecalis* may be provided by the following characteristics: Acid, but no gas from lactose and from mannitol, no reaction with raffinose, no reduction of nitrate to nitrite, production of acid and of casein precipitate in litmus milk. Passing reference is also made to thermo-tolerance and salt-tolerance. This method has been adopted by the World Health Organisation (International Standards for drinking water, 1958). The introduction of a solid medium in order to obtain a pure colony seems preferable to the method applied by LITSKY and MALLMANN (1953) who first use an azide-glucose broth enriched with 0.12 mg/l ethylviolet. A culture is grown at 37° C. As the added ethylviolet checks the growth of Gram-positive spore-forming bacteria, a violet sediment is to be regarded as proof of the presence of streptococci.

In The Netherlands the trend of methodology is entirely in the direction of the growth of pure cultures on a solid medium, which may be looked upon as the continuation of a historical development. In the instructions contained in the "Normblad, Voorschriften voor de bacteriologische keuring van drinkwater, N 1028", which was published in 1942, the examination for the presence of streptococci is described in the same way as it had been by MEERBURG and MASSINK, but with the addition that "if the microscopic examination shows a doubtful picture, or diplococci, the presence of streptococci is further investigated". This was done by growing a sub-culture on lactose-tellurite agar, lacmoid-agar or blood-agar. Characteristic colonies were once more grown in a glucose broth and examined microscopically. With reference to the lacmoid-agar special mention is made of the fact that streptococci are catalase-negative.

A new edition of the "Normblad, Bacteriologisch onderzoek van drinkwater, N 3043", was published in 1956. This spoke for the first time of the "detection of faecal streptococci", thus drawing an express distinction between the streptococci of faecal origin and the rest. The examination is now carried out by adding the sample to a glucose broth containing azide. If a microscopic examination within 48 hours' incubation at 37° C. reveals the presence of streptococci, a sub-culture is grown on tellurite-agar. The test is looked upon as positive if black colonies have developed on this after 48 hours' incubation at 37° C.

In addition the "Normblad" states that these streptococci may be further examined "if required", thus implying that there remains room for doubt. The following characteristics of faecal streptococci are given: diplococci or short chains of cocci; Gram-positive; catalase-negative; growth at 10° C. and at 45° C.; growth with pH =9.6; growth with a concentration of 6.5% sodium chloride; formation of black colonies on tellurite-agar after 48 hours' incubation at 37° C.

The question now arises what kind of *Streptococcus* may be meant. The streptococci have been divided by LANCEFIELD into a number of serological groups denoted by capitals. One of these, group D, is the group of faecal streptococci, the enterococci. These are further subdivided into 4 species, namely:
Str. faecalis (with the variants *zymogenes, liquefaciens* and *haemolyticus*), *Str. faecium, Str. durans,* and *Str. bovis.*

The typical representatives of these 4 types can be distinguished by means of biochemical tests.

From the above it would appear that the first step towards a further identification of the isolated *Streptococcus* strain ought to be a serological classification, which should then be followed up with a biological determination of the species.

However in the normal bacteriological examination of water as carried out in daily practice serological tests cannot very well be applied. Hence these are dispensed with in the instructions of the "Normblad". Group D can also be singled out by means of biochemical tests, i.e. by means of the so-called SHERMAN series, which consists of 8 tests, all of which should be positive in the case of *Str. faecalis.*

This SHERMAN series consists of:

thermo-tolerance (growth at 45° C., and at 10° C.),
salt-tolerance (growth still possible with 6.5% sodium chloride),
alkali-tolerance (growth still possible with pH 9.6),

tolerance with respect to methylene blue (1 : 1000 in skim milk),
thermoresistance (resists a temperature of 60° C. for 30 minutes),
marked reduction in litmus milk,
production of ammonia from peptone (arginine),
growth with 40% bile.

Str. bovis is stated to grow neither at 10° C. nor with pH 9.6 or with 6.5% sodium chloride, while *Str. durans* sometimes does not grow at 45° C. and with pH 9.6.

The "Normblad" N 3043 mentions the first three characteristics of the above list with the additional requirement that black colonies should be formed on tellurite agar which also contains sorbitol and glycerol. This criterion excludes the other enterococci of group D, as only *Str. faecalis* produces distinctly black colonies on tellurite agar. From this it may therefore be concluded that the "Normblad" wishes *Str. faecalis* to be the only indicator organism.

So far everything would be simple enough, but for the fact that in practice one meets with deviations from this scheme. Apart from which there are also problems of a more technical character. Thus DE MOOR (1956) has already pointed out that the tests of the SHERMAN series are actually marginal reactions, so that a negative test is in itself of little value. According to him, growth in a salt broth, for instance, is often hard to judge as it frequently remains feeble. As to growth at pH 9.6, this is not possible in a glucose broth alkalified with NaOH, for the fact that its pH is by no means constant renders the test valueless. What is needed for this is a carefully buffered liquid medium, the pH of which is checked shortly before use (HIRSCH and SHATTOCK (1945), and DE MOOR (1956)). Another drawback is the fact that the blackening on tellurite-agar may show a wide range of transitional shades from pure black to definitely light-grey, which will in certain cases make it difficult to decide whether one can still speak of a black colour. In such a doubtful case one might have recourse to a series of tests based on growth with mannitol, saccharose, raffinose, sorbitol and arabinose. The reactions usually shown by the different types of streptococci of group D are given in table 1.

Unfortunately this method often yields deviating results, which do not fit into the table, so that no solution is found in this way either.

We have therefore tried whether the method of KAUFFMANN and PETERSEN (1956), which is meant for the differentiation of *Enterobacteriaceae*, could also be utilised for enterococci. They used a

TABLE 1.

Characteristics of Enterococci (group D).

	Str. faecalis	*Str. faecalis* var. *zymogenes*	*Str. faecalis* var. *liquefaciens*	*Str. faecium*	*Str. durans*	*Str. bovis*
Growth at 10° C.	+	+	+	+	+	—
,, ,, 45° C.	+	+	+	+	±	±
,, with 6.5% NaCl	+	+	+	+	+	—
,, ,, pH 9.6	+	+	+	+	±	—
,, ,, methylene blue 1 : 1000	+	+	+	+	+	—
Resistance to 60°/30 min.	+	+	+	+	+	—
Marked reduction of litmus milk	+	+	+	±	—	—
Growth with 40% bile	+	+	+	+	+	+
Mannitol	+	+	+	+	±	±
Saccharose	—	+	+	+	—	+
Raffinose	—	—	—	±	—	+
Sorbitol	+	+	+	±	—	—
Arabinose	—	—	—	+	—	±
Potassium tellurite	+	+	+	—	—	—

peptone solution adjusted to pH 7.4, bromthymol blue being added as an indicator. The actual differentiation is carried out by adding organic acids (d-, l-, and i-tartrate, sodium citrate) to the peptone solution.

A positive result is characterized by the fact, that the colour turns from blue to greenish yellow and that after addition of 0.5 ml of a saturated aqueous lead acetate solution to 3 or 4 ml of the medium but little precipitate is produced. If on the other hand, the reaction is negative, the precipitate still measures about 2/3 of the column of fluid after standing for a day.

Our experiments have shown that faecal streptococci also grow in these media. We omitted the bromthymol blue, as the streptococci were found to grow better without. Consequently a positive result can only be ascertained by adding 0.5 ml of a saturated lead acetate solution to 3 or 4 ml of the medium, which must cause but little precipitation. This reaction can be carried out after 48 hours' incubation at 37° C. The original formula gives an incubation period of 5 days, but we found that for streptococci this is not necessary.

The following formula was used by us:

Basic medium:	Bactopeptone, Difco[1]	10 g
	NaOH 0.1 N	8.5 ml
	Distilled water	1000 ml

Add:

1% sodium potassium tartrate, dextro rotatory
or 0.5% tartaric acid, levo rotatory
or 0.5% tartaric acid, inactive
or 1% neutral sodium citrate.
Then adjust pH to 7.4 with 5 N NaOH.

In our experiments with 200 strains of streptococci, further details of which are given below, we found that neither tartaric acid nor sodium potassium tartrate produce a positive reaction. Therefore we only adopted the medium containing 1% neutral sodium citrate in the definitive formula. In doubtful cases, i.e. when the SHERMAN series is positive, but the growth on sorbitol-tellurite-agar does not present a clear picture (grey colonies instead of deep black ones) the conversion of sodium citrate has proved to be a valuable criterion.

OWN INVESTIGATIONS.

The formula used by us for the detection of *Str. faecalis* in drinking-water is as follows: 10 ml water are mixed with 10 ml sodium-azide broth, in the manner described in "Normblad" N 3043, and incubated at 37° C. for 48 hours. If growth occurs, a pure culture is grown on bloodagar. Two tests are then applied simultaneously:

1) A reduced SHERMAN series, *viz.*,
   broth containing 6½% sodium chloride,
   broth with pH 9.6,
   sorbitol-tellurite-agar.
   growth at 45° C. in nutritive broth.
2) Growth in the fluid medium of KAUFFMANN and PETERSEN with 1% sodium citrate, modified in the manner described above.

In this way we examined 200 strains isolated from different ditches and canals in various places of our country. Care was taken to procure polluted water which was very likely to contain streptococci. The results are summarized in table 2.

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[1] The use of this peptone is recommended. A broth rich in trypsine always gives an abundant precipitate with lead acetate (SILBERSTEIN, 1931).

TABLE 2.

Examination of 200 *Streptococcus* strains isolated from water.

Reduced Sherman series	Sorbitol tellurite	Sodium citrate	Number of strains	Conclusion
+	+	+	80	*Str. faecalis*
+ (or partly +)	—	—	65	not group D
+	grey	—	48 1)	not group D
+	grey	+	3 2)	group D
partly +	+	—	3	not group D
+	+	—	1 3)	group D

A number of these strains were serologically examined by Dr. C. E. de Moor with the following results:

1) Three strains taken at random were found to differ serologically from group D.

2) Serologically these strains were found to belong to group D.

3) Though serologically belonging to group D, this strain did not convert citrate and was non-resistant to a heat of 60° C.

As table 2 shows, the conversion of citrate in the modified medium of Kauffmann and Petersen provided us with a clear indication whether an isolated *Streptococcus* strain belonged to the group of faecal streptococci or not. Especially in those cases when the results on sorbitol-tellurite agar did not present a clear picture, the colour not being a deep black, the conversion of citrate helped us to reach a conclusion.

In order to get a clear insight into the reaction of the various types of enterococci to sodium citrate, we examined 55 known enteroccus-strains from the collection of Dr C. E. de Moor. Serologically these strains all belonged to group D. The results are summarized in Table 3.

As this table shows, *Streptococcus faecalis*, and the variants *haemolyticus*, *liquefaciens* and *zymogenes*, are clearly characterized by the reduced Sherman series, including distinct growth with black colonies on sorbitol-tellurite agar, and a positive reaction with sodium citrate. Only 2 strains of *Str. faecalis* var. *haemolyticus* give no citrate-reactions; however, these had not been isolated from water, but from cheese.

Streptococcus bovis and *Str. durans*, are both eliminated by a negative result of the citrate test.

Streptococcus faecium presents a fluctuating picture: of the 8

TABLE 3.

Examination of 55 enterococcus-strains from the collection of the National Institute of Public Health.

Number of strains	Type	Reduced SHERMAN series				Conversion of citrate
		NaCl 6.5%	pH 9.6	Growth 45°C.	S. T. 1)	
21	*Str. faecalis*	+	+	+	+	+
3	*Str. faecalis* variants	+	+	+	+	+
5	*Str. faecalis* var. *haemolyticus*	+	+	+	+	+
2	idem, isolated from cheese	+	+	+	+	—
8	*Str. faecalis* var. *liquefaciens*	+	+	+	+	+
1	idem	+	+	+	±	+
3	*Str. faecalis* var. *zymogenes*	+	+	+	+	+
5	*Str. faecium*	+	+	+	gr.	—
2	idem	+	+	+	gr.	+
1	idem	+	+	+	+	+
2	*Str. durans*	+	—	+	gr.	—
2	*Str. bovis*	—	—	—	O	—

1) S.T. = sorbitol-tellurite agarplate; in this column: + = growth with black colonies, ± = not quite black, gr. = growth with grey colonies, O = growth, but neither grey nor black colonies.

strains examined, 7 grow with grey colonies on the sorbitol-tellurite agar, 5 of these do not react positively in the citrate test, but 2 do; the 8th strain producing distinctly black colonies combined with a positive reaction in the citrate test.

Finally we examined 3 strains of *Str. lactis*, which belongs to the N group. They proved to be citrate-negative.

In view of the above results the question now arises which organism is regarded in the "Normblad" N 3043 as the one that must be found for the result of the test to be called positive.

From the fact that black colonies are required on a sorbitol-tellurite agar, it follows that it is only *Streptococcus faecalis* and its variants that are meant. As the citrate test is used by us to come to a decision when ever these colonies are not definitely deep black, it is not impossible that the result of an examination for "faecal streptococci" by the method prescribed in N 3043 is occasionally put down as positive, whereas it has been caused by *Streptococcus faecium*. This can hardly be an objection, however, as this organism also occurs in the intestinal tubes of mammals (e.g. pigs) and of man.

Summary.

1. An examination of 200 strains of aquatic streptococci has shown that the conversion of citrate provides a good complementary criterion for the detection of faecal streptococci as prescribed in the "Normblad" N 3043. Especially when the result on tellurite agar does not present a clear picture, owing to the colour of the colonies being grey instead of a deep black, the citrate test helps to reach a decision.

2. An examination of 55 known enterococcus-strains has shown that the conversion of sodium citrate gives positive results for: *Streptococcus faecalis* and its variants and for some strains of *Streptococcus faecium*. On the other hand negative results are found for *Streptococcus bovis* en *Streptococcus durans*.

Appendix.

Media.

1. **Sodium azide broth.**

Bactopeptone	30 g
Meat extract	9 g
Glucose	30 g
Sodium chloride	30 g
Sodium azide	0.4 g
Distilled water	1000 ml.

Dissolve by heating and adjust pH of the fluid to 7.2. Filter through paper and sterilize by heating at 110° C. for 15 min.

2. **Sorbitol-tellurite agar.**

Melt 220 ml broth agar and add 2 g sterile glycerol and 1 g sorbitol. Heat for half an hour at 100° C. in a steamer, allow to cool to about 50° C., and add 8 ml of a sterile 1% potassium tellurite solution.

3. **Medium with pH 9.6 according to** Hirsch **and** Shattock.

I. Dissolve:

Dextrose	10 g
Lab Lemco	10 g

Peptone (Evans)	10 g
Sodium chloride	5 g
In tapwater	1000 ml.

Adjust pH to 7.0 and sterilize by heating at 115° C. for 20 minutes.

II. Buffer solution:
Dissolve:

Glycocoll	7.505 g
Sodium chloride	5.85 g

in fresh-boiled glass distilled water 1000 ml. Sterilize by heating at 115°C. for 10 minutes. Mix 6 parts of buffer solution and 4 parts of 0.1 NaOH. Then add 100 ml of this buffer solution to 900 ml of solution I. Adjust pH to 9.8 by adding 1 N NaOH.

Store overnight in the refrigerator (4°C.) until precipitation is complete; then filter through a Seitz filter (E.K.) into small bottles. Store in completely filled and well stoppered bottles.

Acknowledgements.

We are greatly indebted to Dr C. E. de Moor for examination of *Streptococcus* strains and advice.

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GLYOXYLATE INTERMEDIATE OF THE DIRECT OXIDATION OF ACETATE AND GLYCOLATE BY *E. COLI*

by

V. BOLCATO

(Received December 19, 1958).

In preceding papers we have demonstrated that a direct oxidation of acetate appears to occur in yeast (Bolcato *et al.*, 1957) and in *E.coli* (Bolcato *et al.*, 1956a) through the following intermediates:

acetate → acetyl → glycolate → glyoxylate → formaldehyde → formyl →
→ $CO_2 + H_2O$

As to the monocarboxylic acid scheme (MAS), Wong and Ajl (1957), Ajl (1958) and Utter (1958) have pointed to the possibility that glyoxylate might arise, not from acetate and glycolate, but by the action of malate synthetase or isocitritase on malate or isocitrate formed by the tricarboxylic acid cycle (TAC) operative in these cells. If this supposition would prove true, our scheme would lose any validity, as it is the result of the connecting of some reactions concerning the activation of acetate and some others concerning the oxidation of glyoxylate. The possibility that glyoxylate might arise from malate or isocitrate had already been considered by us. In fact, experiments with living yeasts cells (Bolcato and Leggiero, 1958) have demonstrated that glyoxylate could only be obtained from acetate or glycolate and not from succinate, fumarate, malate, and citrate.

The importance that the origin of glyoxylate acquires in our experiments for the validity of the MAS, has led us to study the problem also with living cells of *E.coli*, that are more suitable than the yeast cells for this kind of experiments. In the present paper we report on the oxidation of malate or citrate (instead of isocitrate) either alone or in presence of acetate or glycolate by proliferating and non-proliferating cells of *E.coli*.

METHODS.

Experiments with proliferating cultures.

The avirulent C-14 strain of *E.coli* was used in these experiments. It developed well on a mineral medium containing d,l-malate as sole source of carbon: of the acid only the l-moyety was utilized. Citrate and glycolate were assimilated merely slightly for growth, but were readily oxidized in the medium used in the experiments with non-proliferating cultures. The cells of *E.coli* were grown in two glass tubes provided with a sintered diffuser for aeration. Each tube contained 1000 ml of the following medium: H_2O 1000 ml, K_2HPO_4 9 g, $(NH_4)_2SO_4$ 3 g, $MgSO_4.7H_2O$ 0.3 g, NaCl 1 g, $FeSO_4$, $MnSO_4$ and $ZnSO_4$ in traces, d,l-malate (Merck) 10 g; pH adjusted about 6.2. When the optical density rose to the half of the maximum obtainable, 2.5 g of glycolate (Fluka) or acetate (Merck) were added in one tube and in both 0.3 g of phenylhydrazine oxalate (PO) as trapping agent for glyoxylic acid (table 1-A). Care was taken to avoid blocking the growth of the cells. For the identification of glyoxylic acid and its increasing formation during the experiments the methods described in a preceding paper (BOLCATO *et al.*, 1956b) were used. The results of a typical experiment are assembled in table 1-A.

Experiments with non-proliferating cultures.

The washed cell mass of *E. coli* (22 g w.v.), obtained by following the procedure described elsewhere (BOLCATO *et al.*, 1956a), was suspended in 1600 ml of the following medium: H_2O 900 ml, yeast extract 100 ml, Na_2HPO_4 3 g, KH_2PO_4 1.5 g, $MgSO_4 . 7H_2O$ 0.3 g, d,l-malate (or citrate) 4 g, pH adjusted about 6.2. The suspension was then transferred in equal parts into two glass tubes for aeration. There were added: in one tube 3 g of glycolate and in both tubes 0.3–0.2 g PO salt depending on the pH changes (table 1-B). The results obtained from the experiments with malate alone or associated with acetate or glycolate are assembled in table 1-B).

TABLE 1.

Formation of glyoxylic acid during the oxidation of malate in presence of acetate or glycolate by *E. coli*.

Time	malate			malate + acetate			malate + glycolate		
	pH	PO added g	glyoxylic acid formed mg/l	pH	PO added g	glyoxylic acid formed mg/l	pH	PO added g	glyoxylic acid formed mg/l
A. With proliferating cultures									
13.15	6.6	0.25	—	6.6	0.25	—	6.6	0.25	—
14.45	6.8	0.15	none	6.8	0.15	traces	6.7	0.15	traces
16.00	6.9	—	none	6.9	—	traces	6.8	—	1.0
17.30	7.0	—	none	7.0	—	1.2	6.9	—	2.6
B. With non-proliferating cultures									
11.15	6.3	—	—	6.3	—	—	6.3	—	—
11.55	6.4	0.35	—	6.4	0.35	—	6.4	0.35	—
12.30	6.6	0.35	none	6.6	0.35	none	6.6	0.35	traces
13.00	6.8	0.25	none	6.7	0.25	traces	6.7	0.25	1.2
13.50	7.1	0.25	none	7.0	0.25	traces	7.0	0.25	3.0
14.30	7.4	—	none	7.3	—	1.3	7.3	—	5.0
15.50	7.7	—	none	7.7	—	2.5	7.7	—	12.4
18.30	8.0	—	none	7.9	—	1.8	7.9	—	17.4

Also from citrate alone no formation of glyoxylate could be observed, but only in the presence of acetate or glycolate. The lower amount of glyoxylate obtained from acetate in comparison with that obtained from glycolate is due to the fact that glyoxylate is the direct intermediate of the oxidation of glycolate, while it is the third intermediate of the oxidation of acetate. β-acetylphenylhydrazide and acetophenone, the immediate products of oxidation of acetate, reacting with phenylhydrazine lower its concentration in the medium to a value inadequate to trap glyoxylic acid in sufficient amounts (Bolcato and Leggiero, 1958). We have also observed that glyoxylic acid, also in the form of its phenylhydrazone, is oxidized by the cells with formation of formaldehyde and formyl group as oxidation products (Bolcato, 1959).

CONCLUSION.

From the reported data it is clear that during the respiration of living yeast and *E.coli* cells, glyoxylate cannot be formed from malate or citrate (isocitrate) but only from acetate and glycolate.

This result excludes malate synthetase or isocitritase as active in our experimental conditions and confirms the position of glyoxylate in the MAS, *i.e.* that the acid is an intermediate of the oxidation of acetate and glycolate as it was postulated by us (BOLCATO *et al.*, 1957). The doubt expressed by WONG and AJL (1957), AJL (1958) and UTTER (1958) as to the possible origin in our scheme of glyoxylate from malate or isocitrate may be excluded. Apparently in yeast and in *E.coli* cells the need of glyoxylic acid for the synthesis of C–4 dicarboxylic acid may be satisfied by acetate itself independently of the glyoxylate bypass of the TAC (KORNBERG and MADSEN, 1957; WONG and AJL, 1957; KORNBERG and MADSEN, 1958).

Summary.

From the oxidation of malate and citrate (instead of isocitrate) by living *E.coli* cells no formation of glyoxylate could be observed. On the other hand glyoxylate was always formed in the presence of acetate or glycolate. This result is further proof that glyoxylate is a direct intermediate of the oxidation of acetate and glycolate according to the monocarboxylic acid scheme.

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SOME FURTHER OBSERVATIONS ON SYNERGISM BETWEEN CHLORAMPHENICOL AND POLYMYXIN B IN RELATION TO *SALMONELLA* BACTERIA

by

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(Received February 21, 1959).

In an article in this periodical some years ago (MANTEN and DE NOOY, 1956) we published the results of our experiments concerning the antibacterial effect of some combinations of antibiotics on Salmonellae. Among other things we found that the combination of chloramphenicol and polymyxin showed a marked synergism towards practically all strains tested.

In the same article we contended that the treatment of salmonellosis in man should be aimed at the complete destruction of the bacteria, experience having shown that bacteriostatic substances such as chloramphenicol often effect an incomplete cure, as appears from the occurrence of relapses and the subsequent development of the carrier state. It was further pointed out that the combination of chloramphenicol and polymyxin *in vitro* possesses qualities which might obviate this objection to the use of chloramphenicol.

Since then we have further investigated this synergism with results that will be briefly stated below.

In the experiments referred to above, the favourable interaction between the two antibiotics mentioned had been ascertained by means of the replica method of LEDERBERG (LEDERBERG and LEDERBERG, 1952; ELEK *et al.*, 1953). Results obtained in this way are mainly of a qualitative character and therefore they do not allow of a quantitative judgment. This is only possible if the effect of the antibiotics in question is ascertained in series of exactly known concentrations.

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[1]) With the technical assistance of Miss M. J. WISSE.

RESULTS.

Generally speaking, the interaction between antibacterial substances can be studied in two ways. Most investigators (JAWETZ and GUNNISON, 1952; CHABBERT, 1953; GAIKHORST, 1955; JULIUS and GAIKHORST, 1958; ELEK *et al.*, 1953; MANTEN, 1954, 1956) judge the antibacterial efficacy of these substances by their bactericidal effect. Others (OSKAM, 1956; WEIL and KOTSEVALOV, 1956; FAIRBROTHER and SOUTHALL, 1957; ZWART VOORSPUY and BOKMA, 1958), on the other hand, take the bacteriostasis as their criterion. In our experiments both criteria were applied. The bacteriostasis provided no clear evidence of synergism between polymyxin and chloramphenicol. This part of the investigations was carried out with four strains of *Salmonella schottmuelleri* and one strain of *S. typhimurium*. The ratio in which the two substances were mixed, *i.e.* 1:20, was based on the minimum growth-retarding concentrations of the two antibiotics separately. For polymyxin B-sulphate and chloramphenicol these are 0.2-0.5 μg/ml and 5-10 μg/ml respectively, when the above strains are grown on nutrient agar with pH 7.2-7.5.

As will appear below, there is indeed clear evidence of synergism between these antibiotics if the bactericidal effect is taken as the criterion, so that, in the present case, this would appear to provide a better criterion for judging synergism than bacteriostasis. The replica plating technique is really a judgment of the bactericidal effect and so is the vital counting method adopted in this study. The latter, however, has the advantage of being quantitative.

Briefly, the procedure adopted for the experiments was as follows. The antibiotics were added to tubes containing broth of pH 7.2.-7.5 which had been inoculated with *S. typhimurium*. The tubes were in a waterbath of 37° C., in which they were shaken regularly. They were sampled immediately before the addition of the antibiotics and half an hour, 1 hour, 2, 5 and 24 hours afterwards. The number of living bacteria per μl (mm^3) was counted by means of a pour-plate technique, care being taken to dilute the antibiotics below their bacteriostatic marginal value.

Several concentrations and ratios of the two antibiotics were tried. The results may be summed up thus:

1) The bactericidal effect of polymyxin in quantities of 1 μg/ml and over, is such that suspensions in broth of about 5×10^7 bacteria per ml are generally sterilized within a half to one hour. Concen-

trations under 0.8 μg/ml only effect a bacteriostasis, which grows less as the concentration becomes weaker. Finally a quantity of 0.1 μg/ml only prolongs the lag phase with about 1 or 2 hours.

2) During the first two hours of their action chloramphenicol concentrations of 5—100 μg/ml only effect a bacteriostasis, after which the number of living bacteria slowly decreases. After 24 hours 1-10% of the original number are still alive. Both qualitatively and quantitatively, therefore, the antibacterial effect of chloramphenicol on Salmonellae *in vitro* is inferior to that of polymyxin.

3) The addition of bacteriostatic concentrations of chloramphenicol to bacteriostatic concentrations of polymyxin produces a strong bactericidal effect (synergism). The addition of chloramphenicol to bactericidal concentrations of polymyxin does not increase their bactericidal effect.

4) Within the range investigated, from 5-100 μg/ml, the potentiating effect of chloramphenicol is independent of the concentration.

The result of an experiment in which the bactericidal effect of 0.3 μg/ml polymyxin and that of 10 μg/ml chloramphenicol were compared with the effect of the combination, is shown in Fig. 1. The synergism between the two antibiotics is evident here.

DISCUSSION.

Among other things an ideal remedy against all forms of salmonellosis should combine the following qualities: (1) a strong bactericidal effect, (2) little tendency to cause bacterial resistance, (3) slight clinical toxicity and (4) the power to reach the bacteria anywhere in the body.

Judging from what has been observed *in vitro* or from what can be theoretically deduced, the combination of chloramphenicol and polymyxin amply meets the requirements under (a) and (b): but *in vitro* observations can only provide a partial solution of the problem whether the qualities mentioned sub (c) and (d) can also be ascribed to the combination.

The main clinical drawbacks of polymyxin are inherent in its lack of power to penetrate the various tissues and cells, so that bacteria hiding there may escape its effect, or they are of a toxicological nature. The latter can be obviated by administration per os, as polymyxin is hardly resorbed at all from the alimentary canal and does not cause demonstrable blood levels. As was stated before,

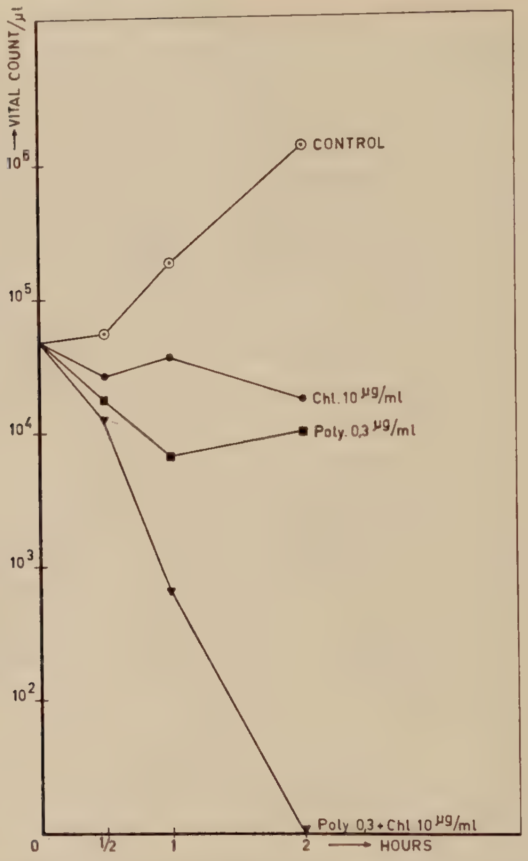

Fig. 1. Synergism between polymyxin and chloramphenicol on a level of 0.3 µg/ml of the former antibiotic. Note the rapid bactericidal action of the combination.

chloramphenicol alone is often ineffective, because usually it brings about only a bacteriostasis. The fact established by these experiments, that under test conditions a quantity of as little as 0.3 µg/ml polymyxin together with 10 µg/ml chloramphenicol has a strong bactericidal effect *in vitro*, leads one to expect that the combination will obviate these objections to some extent.

The data collected by one of us (de N.) with regard to the clinical treatment with a combination of polymyxin and chloramphenicol are as yet not very extensive. Nevertheless, the results, especially those obtained with "recent carriers" of *Salmonella* organisms, who only carry these bacteria in the intestinal tract, justify some optimism.

Summary.

The synergism between chloramphenicol and polymyxin B sulphate, earlier noticed using a replica technique, has been reexamined applying quantitative methods. Vital count experiments in suspensions of *Salmonella typhimurium* in broth have revealed that addition of bacteriostatic concentrations of chloramphenicol in a range of 5-100 μg/ml to bacteriostatic concentrations of polymyxin (0.1-0.9 μg/ml) produce a strong bactericidal effect (synergism). Combinations of bactericidal concentrations of polymyxin (1 μg/ml and higher) with chloramphenicol also exert a very high degree of bactericidal activity, though no more than with polymyxin alone (no synergism).

Chloramphenicol therapy sustained with polymyxin, may be of therapeutic value in the eradication of stubborn enteral *Salmonella* infections.

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VERGLEICHENDE UNTERSUCHUNGEN ZUR BESTIMMUNG DER PROTEOLYTISCHEN KEIME

von

K. CSABA und **I. NIKODEMUSZ**

(Eingegangen am 26. Januar 1959).

Es ergibt sich in den letzten Jahren ein wachsendes Interesse für die Zahlung proteolytischer Bakterien im Bezug auf Nahrungsvergiftungen durch nicht-spezifische Krankheit erregende Bakterien (1, 2, 3, 4, 8, 9). Eine Publikation von Mossel und de Bruin (5) in welcher für die Zahlungen Frazier's Gelatine Agar benutzt wurde, zog in diesem Zusammenhange unsere Aufmerksamkeit. Von uns so wie von andern wird dazu Milchagar angewendet (6, 10), aber diese Methode hat einige Nachteile.

1. Auf Milchagarplatten wachsen die Milchsäurebakterien ziemlich gut, deshalb kann man einige Milchprodukte (Yoghurt, Kefir, Sahne, Butter) auf Grund ihrer Gesamtkeimzahl nicht beurteilen.

2. Die Milchsäurebakterien und auch andere laktose-positive Keime zeigen auf Milchplatten eine sogenannte Pseudoproteolyse, die unter gewissen Umständen mit dem echten Eiweissabbau leicht verwechselbar ist.

Es kam uns darum wünschenswert vor unsere Methode mit der von Mossel und de Bruin zu vergleichen.

Es ist bekannt dass die proteolytischen Keime zu vielen Gruppen (aerobe Sporenbildner, Clostridia, Darmbakterien, chromogene Bakterien) gehören. Eben darum benötigt man zu ihrer völligen Identifikation eine ziemlich lange Zeit. Ihre Zahlung, die auf den Nachweis ihrer proteolytischen Fähigkeit beruht, ist aber wegen ihrer Signifikanz notwendig.

Ihre Bestimmung wird in der täglichen Routine-Diagnostik im Allgemeinen auf zweierlei Arten durchgeführt: 1. durch Spaltung des Milcheiweisses, 2. durch Spaltung der Gelatine. Nach unserem Wissen ist die zweite Methode mehr verbreitet, wahrscheinlich darum, weil auf Grund des Milcheiweissabbaus die Proteolyse von der sogenanten Pseudoproteolyse schwer abgrenzbar ist. Um die Frage zu entscheiden welche Methode für die Beurteilung der Lebensmittelprodukte auf Grund ihres saprophytischen Keimgehalts besser geeignet ist, führten wir Serien-Untersuchungen mit verschiedenen Lebensmitteln durch, um unsere diesbezüglichen Kentnisse zu ergänzen.

METHODIK.

Zwei Methoden wurden parallel angewendet. Von den Lebensmittelmustern in einer Verdünnung von 10^1, 10^2, 10^3, 10^4, 10^5 wurden je 0,5 ml in Platten gegossen 1. mit Milch-Agar nach POLÓNYI (6, 10) (80 %-Nähragar, 20 % sterilisierte Magermilch). Der Eiweissabbau ist direkt ablesbar. 2. mit 1 %-igen Gelatine-Agar nach FRAZIER-MOSSEL (5). Der Gelatineabbau wird nach Benützung von Sublimat-Reagens abgelesen.

Bevor wir unsere Untersuchungen begannen, hielten wir es für wichtig einige eiweissspaltende Mikroben in milch- und gelatinehaltigem Milieu parallel zu beobachten. Diese Stämme wurden aus verschiedenen Lebensmittelprodukten isoliert. Unsere Ergebnisse sind im folgenden zusammengefasst: 43 *Bac. cereus*, 4 *Bac. subtilis*, 3 *Bac. mycoides*, 3 *Ps. pyocyanea*, 3 *Ps. fluorescens*, 2 *B.proteus* und 11 nicht identifizierte aerobe Sporenbildner-Stämme spalteten das Kasein und die Gelatine in gleicher Weise. 1 *B.prodigiosus*-Stamm spaltete nur die Gelatine. Nur auf Milch-Agarplatten wurde die Proteolyse (Aufklärung) von 5 *Streptococcus lactis* und 2 *Oidium lactis* Stämme beobachtet. 10 *Lactobacillus*-Stämme, 5 *Bac. megatherium*, und 15 weitere aerobe Sporenbildner zeigten weder kaseolytische, noch gelatinolytische Aktivität. Wir konnten mit den von uns isolierten *Staphylococcus aureus* und *Staphylococcus albus*-Stämme kein übereinstimmendes Bild bekommen. Zur Beobachtung des Eiweissabbaus dieser Stämme sind noch weitere Untersuchungen notwendig. Im Allgemeinen spalten sie weder Kasein noch Gelatine in Agarnährboden.

Beide Nährböden wurden mit verschiedenen Verdünnungen von standard Stämmen verglichen um die Unterschiede zwischen den Keimzahlen beider Nährböden festzustellen. Mit den Suspenzionen von je 2 *Staphylococcus aureus*, 2 *E.coli*, 2 *Aerobacter aerogenes*, 2 *B.proteus*, 1 *B.prodigiosus* Stämme wurden Platten ausgegossen.

Es wurde festgestellt, dass auf Milch- u. Gelatine-Platten fast die gleiche Menge von Kolonien zu beobachten waren. Die Differenz war innerhalb der biologischen Streuung. Unsere vergleichende Untersuchungsserien wurden mit den folgenden Lebensmittelprodukten durchgeführt:

Milch	60 Muster
Sahne	60 ,,
Milchprodukte (Yoghurt, Kefir, u.s.w.)	30 ,,
Kaltküchenprodukte	80 ,,
Wurstwaren	60 ,,
Presswurst	30 ,,
Trockenmilch	50 ,,

Wir mussten auch daran denken, dass unter den Mustern auch solche Produkte seien, die eine sogenannte Edelflora enthalten.

Mit unseren Experimenten wollten wir auf folgende Fragen Antwort erhalten. 1. Gibt es einen Unterschied zwischen der Gesamtkeimzahl der zwei Nährböden und was ist die Ursache der etwaigen Differenz. 2. Gibt es einen Unterschied zwischen der proteolytischen Zahl und was ist die Ursache dieser Differenz.

Bei der Summierung unserer Ergebnisse wurde die folgende Methodik angewendet: Die prozentuelle Differenz der Gesamtkeimzahl und des proteolytischen Index auf Gelatine-Nährböden wurde immer verglichen mit den auf Milch-Agar erhaltenen Werten und von den Ergebnissen der Durchschnittwert berechnet. Die Tabelle zeigt die Durchschnittwert der prozentuellen Abweichung der auf Gelatine-Nährböden erhaltenen Gesamtkeimzahl und der proteolytischen Zahl im Verhältnis zu den auf Milchnährböden erhaltenen Werten.

TABELLE

Material	Abweichung in der Gesamtkeimzahl	Abweichung in der proteolytischen Zahl
Milch	— 13%	— 15%
Sahne	— 86%	— 3%
Verschiedene Milchprodukte (Yoghurt, Kefir)	— 73%	— 42%
Verschiedene Kaltküchenprodukte	— 15%	— 19%
Wurstwaren	— 60%	— 4%
Presswurst	— 83%	+ 5%
Trockenmilch	— 10%	— 1%

AUSWERTUNG DER UNTERSUCHUNGSERGEBNISSE.

Wenn wir unsere Ergebnisse und unsere Beobachtungen zusammenfassen, erhalten wir auf die gestellten Fragen folgende Antwort:
1. Die Differenz zwischen der auf den zwei Nährböden erhaltenen Gesamtkeimzahl, liegt innerhalb der Streuung der verwendeten Methode wenn die Bakterien ebenso gut wachsen auf Gelatine Nährboden wie auf Milchagar. Bei Sahne, Wurstwaren, Presswurst und Milchprodukten konnte jedoch auf Gelatine-Nährböden eine 60–86 %-ige Keimzahlverminderung beobachtet werden. Die Ursache diese Phänomens scheint darin zu liegen, dass sich die Keime der Lactobazillus-Gruppe auf Gelatine-Platten nicht vermehren. Es wäre vielleicht besser den Gesamtkeimgehalt der Lebensmittel anstatt auf Milch-Nährböden auf Gelatine-Nährböden zu bestimmen, denn auf Milch-Agar ist die bakteriologische Beurteilung auf Grund der Gesamtkeimzahl nicht möglich. Wir müssen noch erwähnen, dass bei den Milchmuster keine Unterschiede vorhanden waren, wahrscheinlich weil die Pasteurisierung die Edelflora vernichtet hatte.
2. Bei der Bestimmung der proteolytischen Keime von Milch-, Sahne-, Wurst- und Presswurst-Mustern konnte auf Gelatine und Milch enthaltenden Nährmedien kein wesentlicher Unterschied beobachtet werden. Bei der Untersuchung von Kaltküchenprodukten war der Unterschied schon beträchtlich und bei Milchprodukten konnte auf Gelatine-Agar eine ausgeprägte Erniedrigung der Zahl der proteolytischen Keime nachgewiesen werden. Diese Differenz betrug 42 %. Die Ursache dieses Phänomens liegt wahrscheinlich darin, dass die Edelflora von Yoghurt und Kefir auf Milchplatten eine sogenannte Pseudoproteolyse zeigt, die unter gewissen Umständen mit der echten Proteolyse leicht verwechselbar ist. Die Lactobazillen können sich auf Gelatine-Nährböden nicht vermehren und einige Keime können nur Kasein spalten, aber Gelatine nicht [*Micrococcus caseolyticus* (7)].

Die echten proteolytischen Keime sind im allgemeinen anspruchslos und können auf beiden Nährböden gut gedeihen.

Wir müssen noch erwähnen, dass nach den ungarischen lebensmittelbakteriologischen Vorschriften die Bestimmung der Gesamt- und proteolytischen Flora auf Milch-Agarnährböden durchgeführt wird, aber die Gesamtkeimzahl der Edelflora enthaltenden Lebensmittelprodukte nicht in Betracht kommt (6).

Zusammenfassung.

Aus unseren Versuchsergebnissen kann man feststellen, dass Milch-Agar und Gelatine-Agar bei der Keimzahlbestimmung ungefähr gleichwertig sind. Vorteil des Milch-Agars ist, dass die proteolytischen Keime sofort, ohne Reagens ablesbar sind. Die Differenzierung der proteolytischen und pseudoproteolytischen Keime macht jedoch Schwierigkeiten. Der Vorteil des Gelatine-Agars ist, dass sich die Milchsäurebakterien auf diesem nicht vermehren und die Beurteilung der Lactobazillen enthaltenden Lebensmittelprodukte auf Grund ihrer Gesamtkeimzahl auch durchführbar ist. Als weiterer Vorteil ist zu erwähnen dass die Pseudoproteolyse auf Gelatine-Platten nicht bemerkbar ist.

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UNTERSUCHUNGEN ÜBER DIE PHYSIOLOGIE DER WEINHEFEN. VIII. MITTEILUNG

ÜBER DIE WIRKUNG VERSCHIEDENER ZUCKERARTEN, ZUCKERALKOHOLE, ZUCKERKARBONSÄUREN UND GLUCOSIDE AUF DIE ANAEROBE GÄRUNGSHEMMUNG BEI DER „FENDANT"-WEINHEFE (*SACCHAROMYCES CARLSBERGENSIS* HANSEN) IN BERNSTEINSÄURE-KALIUMSUCCINAT-PUFFER[1])

von

T. WIKÉN und **N. PFENNIG**[2])

(Eingegangen am 4. Februar 1959).

Bei der alkoholischen Gärung lebender Wein- und Brauereihefezellen tritt unter bestimmten Bedingungen ein Hemmungseffekt auf, der u. a. durch folgende Merkmale gekennzeichnet ist (49, 50, 56, 57, 58, 59, 60):

1. In ausgeprägter Form kommt die Hemmung nur bei „ruhenden" Zellen aus jungen Hefekulturen vor. Bei Zellen aus alten Kulturen kann sie gar nicht oder höchstens in sehr geringem Ausmasse festgestellt werden.

2. Die Hemmung wird durch Begasung der Zellen mit O_2-freiem Stickstoff, Wasserstoff, Kohlendioxyd oder Argon hervorgerufen, wobei sie mit zunehmender Begasungsdauer grösser wird.

3. Die Hemmung ist reversibel, indem sie sich durch Zusatz von molekularem Sauerstoff vor dem Beimischen des Zuckers vollständig oder fast vollständig beseitigen lässt. Das Ausmass der Reaktivierung der Zymase hängt vom O_2-Gehalt der Gasphase der

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[1]) I, II, III, IV, V, VI und VII Mitteilung: Antonie van Leeuwenhoek 17, 209, 1951; 18, 31, 1952; 18, 293, 1952; 19, 279, 1953; 20, 385, 1954; 21, 337, 1955; 23, 113, 1957.

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gärenden Systeme ab, indem die Gärgeschwindigkeit im Bereich von 0,005 bis 10 à 20 Volumen-% Sauerstoff zunimmt.

4. Der Hemmungseffekt ist für Gärungen im Bernsteinsäure-Succinat-Puffersystem spezifisch, wobei es gleichgültig ist, ob dieses Natrium- oder Kaliumsuccinat enthält. In dest. Wasser sowie Pufferlösungen von Citronensäure-Citrat, Fumarsäure-Fumarat, DL-Äpfelsäure-Malat und Essigsäure-Acetat kann keine Hemmung nachgewiesen werden. In DL-Weinsäure-Tartrat-Puffer tritt ein ähnlicher Effekt auf. Dieser ist aber äusserst gering und verschwindet bei steigender Konzentration des Puffers.

5. Die Tatsache, dass die Aufhebung der anaerob auftretenden Gärungshemmung bereits durch minimale O_2-Mengen (0,005 Volumen-% Sauerstoff in der Gasphase der Gärkolben entsprechen 2,5 - 3 μl O_2 pro 300 mg feuchte Hefe oder ca. 60 mg Hefetrockensubstanz) induziert werden kann, deutet darauf hin, dass es sich um eine Inaktivierung bzw. Reaktivierung einer oder mehrerer Komponenten des Zymasekomplexes oder der damit gekoppelten Enzymsysteme handelt.

6. In Gärungen, welche mit Gemischen von Zellen aus jungen bzw. alten Hefekulturen angesetzt werden, behalten die verschieden alten Zellen ihre individuellen Gäreigenschaften bei. Dies deutet darauf hin, dass der Hemmungseffekt auf eine Inaktivierung von Zellbestandteilen zurückzuführen ist, welche sich nicht von den intakten Zellstrukturen trennen lassen bzw. nicht vom alten Zellmaterial ins Medium ausgeschieden und vom jungen Zellmaterial aus diesem aufgenommen werden können.

7. Die Eigenschaft, in Bernsteinsäure-Succinat-Puffer unter anaeroben Bedingungen eine ausgeprägte Gärungshemmung aufzuweisen, ist an einen bestimmten Abschnitt der Vermehrungskurve, d.h. an eine bestimmte Phase in der Entwicklung der Hefekulturen gebunden. Sie ist von den im Laufe der Vermehrung der Hefezellen stattfindenden Veränderungen in der Zusammensetzung des Kultursubstrats unabhängig.

8. Die Hemmung ist eine dem PASTEUR-MEYERHOF-Effekt entgegengesetzte Erscheinung, indem die Gärgeschwindigkeit bei abnehmender O_2-Konzentration kleiner wird. Unseres Erachtens ist es aber trotzdem unangebracht, sie als einen „negativen PASTEUR-MEYERHOF-Effekt" aufzufassen oder auch nur zu bezeichnen, und dies nicht nur deshalb, weil der Mechanismus des Hemmungseffekts unbekannt ist, sondern auch im Hinblick auf die Tatsache, dass die

zahlreichen Mechanismen, auf welche der PASTEUR-MEYERHOF-Effekt im Laufe einiger Jahrzehnte zurückgeführt worden ist, noch heute aus guten Gründen stark umstritten sind (vgl. HOOGERHEIDE (19), DIXON (16), BURK (8), JOHNSON (20), LIPMANN (23, 24), STERN und MELNICK (43), MEYERHOF (29), SUMNER und SOMERS (45), S. 374, DICKENS (13), AISENBERG und POTTER (1), AISENBERG, REINAFARJE und POTTER (2), CLIFTON (11), S. 135, LAWS und STICKLAND (22)).

9. Der Hemmungseffekt ist von der Konzentration des Bernsteinsäure-Succinat-Systems abhängig, indem er unter den gewählten experimentellen Bedingungen in 0,04 M Lösung des Puffers kaum sichtbar ist und erst im Konzentrationsbereich 0,08 - 0,1 M deutlich zum Vorschein kommt.

10. Da das Ausmass der Hemmung bei konstantem pH-Wert (4,9 ± 0,04) einerseits von der O_2-Konzentration, anderseits von der Stärke der Bernsteinsäure-Succinat -Lösung abhängig ist, kann die gleiche Vergrösserung der Gärungshemmung sowohl durch Erhöhung der Bernsteinsäure-Succinat-Konzentration bei konstantem O_2-Partialdruck, als auch durch Erniedrigung des O_2-Partialdrucks bei konstanter Konzentration des Puffers bewirkt werden.

11. Die alkoholische Gärung der Hefen ist nach klassischen Begriffen ein anoxydativer oder anoxybiontischer Prozess, der sich unter anaeroben Bedingungen glatt abspielen kann. In Bernsteinsäure-Succinat-Puffer bestimmter Konzentrationen trifft dies aber nur im Falle des aus alten Kulturen gewonnenen Hefematerials zu. Die aus jungen Kulturen stammenden Zellen müssen in Anwesenheit des erwähnten Puffers in bezug auf die alkoholische Gärung als mikroaerophil bis aerob bezeichnet werden, indem sich dieser Prozess mit normaler Geschwindigkeit nur in Gegenwart minimaler bis grosser O_2-Mengen abspielt.

Gemäss unserer vorläufigen Arbeitshypothese (49) ist der Hemmungseffekt darauf zurückzuführen, dass im Laufe der Begasung des in Bernsteinsäure-Succinat-Puffer aufgeschwemmten jungen Hefematerials mit O_2-freiem Stickstoff, Argon, Wasserstoff oder Kohlendioxyd irgendwelche Zentren in den Zellstrukturen oder aktiven Bezirken der Enzymoberflächen derart verändert werden, dass die normalen Komplexe einer oder mehrerer Zymasekomponenten mit dem Zucker oder einem bis mehreren intermediären Gärprodukten nicht gebildet werden können. Wie bereits erwähnt, ist das Ausmass dieser Blockierung, d.h. die Zahl inaktivierter Zentren, bei konstantem pH vom O_2-Partialdruck in der Gasphase und von der Konzentration der Bernsteinsäure-Succinat-Lösung im Augenblick des Zuckerzusatzes abhängig. Wir hielten es nun für wichtig festzustellen, ob die anaerobe Gärungshemmung aufgehoben werden kann durch Zuckerarten, Zuckeralkohole, Zuckerkarbonsäuren und Glucoside, welche den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff, d.h. aerob, beigemischt werden.

In den früheren Versuchen (49, 56, 57, 58, 59, 60) diente stets Glucose als Gärsubstrat. Es erschien uns bedeutungsvoll zu prüfen, ob der Hemmungseffekt auch bei anderen vergärbaren Verbindungen auftritt und mithin genereller Natur ist, oder ob er auf die Vergärung der Glucose beschränkt ist.

Es ist ferner von Interesse festzustellen, ob nur diejenigen Verbindungen, welche unter aeroben oder anaeroben Bedingungen vergoren werden, zur Aufhebung des Hemmungseffekts befähigt sind, oder ob auch unvergärbare Stoffe dieses Vermögen besitzen.

Wie unten ausgeführt wird, lässt sich die anaerobe Gärungshemmung durch bestimmte, den Hefesuspensionen aerob beigemischte Zuckerarten aufheben. Aus verschiedenen Gründen, und zwar u. a. im Hinblick auf die oben erwähnte Arbeitshypothese, wollten wir auch die Abhängigkeit der Hemmungsaufhebung von der Menge des zugesetzten Zuckers ermitteln. Es war uns schliesslich wichtig zu prüfen, ob Zucker, der bei niedriger Temperatur (0,1° C.) vor der Begasung aerob zugesetzt wird, ebenfalls eine Beseitigung der Gärungshemmung bewirken kann.

METHODIK.

Die Methoden, welche bei der Züchtung der Hefe, Herstellung der Suspensionen ruhender Zellen und Durchführung der Gärversuche zur Verwendung kamen, wurden bereits ausführlich beschrieben (51, 55, 57, 62). Hier sei erwähnt, dass das Hefematerial in Substrat C ohne Zusatz von Wuchsstoffen gewonnen wurde. Dieses Substrat, dessen pH-Wert nach dem Sterilisieren 4,8 - 5,0 beträgt, enthält Mineralsalze und Kaliumcitrat-Puffer sowie vitaminfreies Caseinhydrolysat und Glucose (51, 62). Sämtliche Experimente wurden mit junger Hefe durchgeführt. Beim Ernten des Zellmaterials betrug das Alter der Kulturen 15½ Std. (Abb. 3 A und 5 A), bzw. 16 Std. (Abb. 4 A, 4 C und 4 D sowie 6 D), 16½ Std. (Abb. 1 A - 1 D, 2 A - 2 D, 3 C und 4 B sowie 5 B, 5 D und 5 E) und 17½ Std. (Abb. 3 B, 5 C und 6 A - 6 C sowie 7 und 8).

Die Kohlendioxydproduktion der 2mal in dest. Wasser und 1mal in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer (pH = 4,9) gewaschenen Hefezellen wurde volumetrisch in der Apparatur nach von Euler, Myrbäck, Nilsson und Alm (36) bei 25° C. gemessen.

Die Hefesuspensionen sowie die Lösungen der zu prüfenden Zuckerarten, Zuckeralkohole, Zuckerkarbonsäuren und Glucoside wurden durchgehend mit dem erwähnten Bernsteinsäure-Kaliumsuccinat-Puffer bereitet.

Die anaeroben Gärungen wurden mit Hefesuspensionen durchgeführt, welche entweder während 120 Min. in strömendem O_2-freiem Stickstoff (Abb. 1 A - 1 D, 2A - 2 D, 3 B und 3 C sowie 5 A - 5 E, 6 A - 6 D, 7 und 8) oder während 100 Min. in strömendem und anschliessend während 20 Min. in ruhendem O_2-freiem Stickstoff (Abb. 3 A sowie 4 A - 4 D) geschüttelt worden waren. Die bei den aeroben Gärungen verwendeten Zellsuspensionen wurden durchgehend während 120 Min. in ruhender Luft geschüttelt (Abb. 4 A - 4 D, 5 A - 5 E sowie 6 A - 6 C und 8).

Die Gärkolben, deren Gesamtvolumen je etwa 50 ml betrug, enthielten in sämtlichen Versuchen 2,0 ml Zellsuspension im Hauptraum und 1,0 ml Lösung der zu vergärenden Verbindung in der seitlich eingeschliffenen drehbaren Ampulle. Die Menge frischer Hefe betrug normalerweise 300 mg pro Gärgefäss (betr. Ausnahme siehe Legende der Abb. 7).

In den Versuchen über die hemmungsaufhebende Wirkung von aerob, d. h. vor der Begasung mit O_2-freiem Stickstoff zugesetzten Zuckerarten, Zuckeralkoholen, Zuckerkarbonsäuren und Glucosiden kamen 50 mg der zu prüfenden Verbindungen zur Verwendung. Die anaerob, d.h. nach der Begasung mit O_2-freiem Stickstoff beigemischte Glucosemenge betrug 200 mg (Abb. 1 A - 1 D sowie 2 A - 2 D und 3 A - 3 C).

Bei der Prüfung der aeroben und anaeroben Vergärbarkeit der verschiedenen Verbindungen setzten wir Glykogen, α- und β-Dextrin (Abb. 4 D) sowie die neutralisierten Lactone der Gluconsäure (Abb. 5 C), der Glucoheptonsäure, Galactonsäure, Gulonsäure, Arabonsäure und Ribonsäure (Abb. 5 D) in einer Menge von 50 mg zu. Sämtliche übrigen Verbindungen lagen in den Versuchen über die Vergärbarkeit (Abb. 4 A - 4 D sowie 5 A - 5 E) in einer Menge von 200 mg pro Gärkolben vor. In den in Abb. 6, 7 und 8 dargestellten Messreihen betrugen die von der vorbehandelten Hefe zu vergärenden Glucosemengen durchgehend 200 mg pro Gärgefäss. Die in den gleichen Versuchen zur Aufhebung der Gärungshemmung aerob zugesetzten Glucosemengen gehen aus den betreffenden Legenden hervor.

Folgende Verbindungen wurden auf ihr Vermögen, bei aerobem Zusatz die anaerobe Gärungshemmung aufzuheben, sowie auf ihre Vergärbarkeit unter aeroben und anaeroben Bedingungen bzw. auf Vorhandensein der erwähnten Gärungshemmung geprüft:

Pentosen: D-Arabinose (1), L-Arabinose (1), D-Xylose (1), L-Xylose (1), D-Ribose (1, 4).

Methylpentosen: L-Rhamnose (1), L-Fucose (1).

Hexosen: D-Glucose (1), D-Mannose (1), D-Galactose (1, 4). D-Fructose (1), L-Sorbose (1).

Disaccharide: Maltose (1), Trehalose (1), Cellobiose (2), Saccharose (2), Turanose (2), Lactose (3), Melibiose (2).

Trisaccharide: Melezitose (2), Raffinose (1).

Polysaccharide: Glykogen (1), Inulin (2), α-Dextrin (5), β-Dextrin (5).

Zuckeralkohole: Glycerin (3), Erythrit (2), L-Arabit (2), Adonit (2), Dulcit (2), D-Mannit (1), D-Sorbit (1).

Zuckerkarbonsäuren: D-Arabonsäure- λ-lacton (1), D-Arabonsaures Calcium (1), D-Ribonsäure-λ-lacton (1), D-Gluconsäure-λ- und -δ-lacton (1), D-Galactonsäure-λ-lacton (1), Gulonsäure-lacton (2), α-Glucoheptonsäure-lacton (2), Glucoheptonsaures Calcium (2),

Uronsäuren: D-Glucuronsäure-λ-lacton (1), D-Galacturonsäure (1).

Glucoside: β-Methyl-D-Xylosid (2), α-Methyl-D-Glucosid (2), α-Methyl-D-Mannosid (2).

Die mit (1) bzw. (2), (3), (4) und (5) bezeichneten Präparate bezogen wir von Hoffmann-La Roche (Basel) bzw. Pfanstiehl Chemical Co. (Waukegan, Ill.), Merck (Darmstadt), Mann Research Laboratories und Herrn Professor Dr. K. MYRBÄCK (Stockholm). Zur Herstellung der Pufferlösung dienten Bernsteinsäure p. a. und Kaliumhydroxyd puriss. p.a . von der Firma Merck (Darmstadt).

VERSUCHSERGEBNISSE.

In einer Reihe von elf Versuchen wurde zunächst geprüft, ob Zuckerarten, Zuckeralkohole, Zuckerkarbonsäuren und Glucoside die anaerobe Gärungshemmung aufzuheben vermögen, wenn sie den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff, d. h. aerob, beigemischt werden. Wie bereits erwähnt, wurden durchgehend 50 mg dieser zu prüfenden Verbindungen zugesetzt, während die nach Begasung mit O_2-freiem Stickstoff, d. h. anaerob, zu vergärende Glucosemenge 200 mg betrug. Die Ergebnisse der einzelnen Versuche, welche in Abb. 1 A - 1 D, 2 A - 2 D und 3 A - 3 C vorliegen, lassen sich wie folgt zusammenfassen:

D-Glucose (9 Versuche) sowie D-Fructose, D-Mannose und Saccharose (je 2 Versuche) sind durch ein ausserordentlich starkes Vermögen, die Hemmung aufzuheben, gekennzeichnet. Unter den gewählten experimentellen Bedingungen ist dieses Vermögen für die erwähnten Zuckerarten gleich gross, da die betreffenden Gärkurven praktisch identisch sind (Abb. 1 A, 1 B und 2 C).

Eine sehr starke Wirkung zeigt ferner Raffinose (2 Versuche). Sie ist gleich gross (Abb. 1 C) oder ein bisschen kleiner (Abb. 2 C) als diejenige der D-Glucose, D-Fructose, D-Mannose und Saccharose.

Eine verhältnismässig starke hemmungsaufhebende Fähigkeit besitzen weiterhin D-Galactose und D-Ribose (je 3 Versuche). Dabei zeigen die Präparate der Firma Hoffmann-La Roche die gleiche Wirkung wie diejenigen („assayed"), welche von Mann Research Laboratories bezogen wurden (Abb. 3 C).

Von den Zuckerarten weist auch Maltose (2 Versuche) eine deutliche hemmungsbeseitigende Wirkung auf, während L-Arabinose, L-Rhamnose und Cellobiose (je 2 Versuche) sowie L-Xylose, L-Fucose und Trehalose (je 2 Versuche) nur durch einen verhältnismässig schwachen aufhebenden Effekt gekennzeichnet sind. Bei D-Xylose, L-Sorbose, Lactose und Melibiose (je 2 Versuche) lässt sich keine oder höchstens eine geringe Aufhebung der anaeroben Gärungshemmung feststellen, während D-Arabinose, Turanose und Melezitose (je 2 Versuche) vollständig inaktiv sind.

Von den geprüften Polysacchariden vermochte β-Dextrin (1 Versuch) die anaerobe Gärungshemmung ein wenig zu beseitigen, während Glykogen (2 Versuche) keine oder höchstens eine sehr geringe Aufhebung bewirken konnte und α-Dextrin sowie Inulin (je 1 Versuch) völlig inaktiv waren.

Die Zuckeralkohole rufen eine kleine Hemmungsaufhebung hervor, wobei Adonit, D-Mannit und D-Sorbit am aktivsten sind, während die Wirkung des L-Arabits, Dulcits, Glycerins und Erythrits geringer ist und in der genannten Reihenfolge abnimmt (je 1 Versuch). Die beiden letzten Polyalkohole können in der Tat als praktisch inaktiv bezeichnet werden.

Von den Zuckerkarbonsäuren zeigt nur die Glucoheptonsäure als

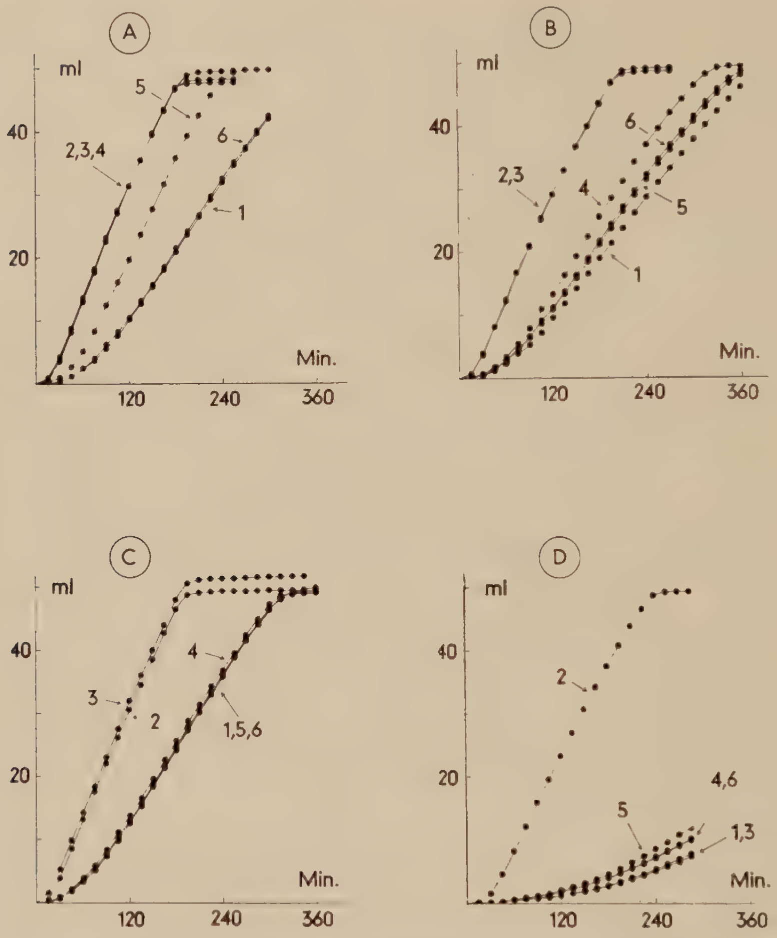


Abb. 1. Aufhebung der unter anaeroben Bedingungen in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer auftretenden Hemmung der Vergärung der Glucose durch Zuckerarten, welche den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff, d.h. aerob, beigemischt werden.

A: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Glucose,
3. Fructose,
4. Mannose,
5. Galactose Hoffmann-La Roche,
6. Sorbose.

B: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Glucose,
3. Saccharose,
4. Maltose,
5. Lactose,
6. Cellobiose.

C: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Glucose,
3. Raffinose,
4. Melibiose,
5. Melezitose,
6. Turanose.

D: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Glucose,
3. Glykogen,
4. Trehalose,
5. Rhamnose,
6. Fucose.

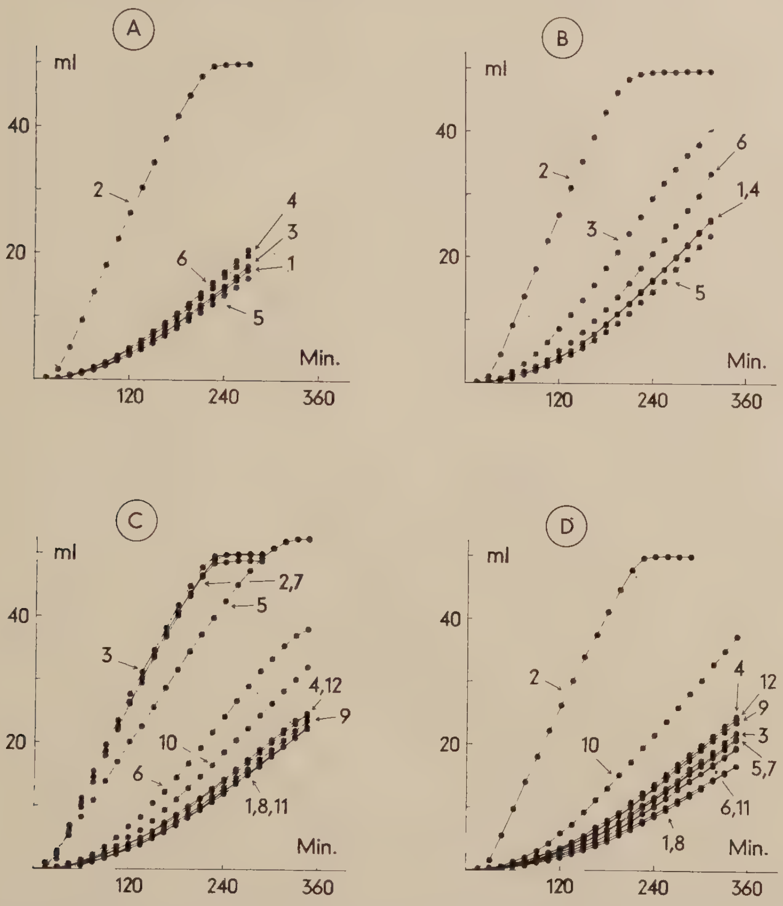


Abb. 2. Aufhebung der unter anaeroben Bedingungen in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer auftretenden Hemmung der Vergärung der Glucose durch Zuckerarten, welche den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff, d.h. aerob, beigemischt werden.

A: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Glucose,
3. D(+)-Xylose,
4. L(—)-Xylose,
5. D(—)-Arabinose,
6. L(+)-Arabinose.

B: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Glucose,
3. D(—)-Ribose Hoffmann-La Roche,
4. Inulin,
5. α–Dextrin,
6. β–Dextrin.

C: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Glucose,
3. Saccharose,
4. Melibiose,
5. Raffinose,
6. Galactose Hoffmann-La Roche,
7. Fructose,
8. Melezitose,
9. Turanose,
10. Maltose,
11. Lactose,
12. Trehalose.

D: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Mannose,
3. Sorbose,
4. Rhamnose,
5. Fucose,
6. D(+)-Xylose,
7. L(—)-Xylose,
8. D(—)-Arabinose,
9. L(+)-Arabinose,
10. D(—)-Ribose Hoffmann-La Roche,
11. Glykogen,
12. Cellobiose.

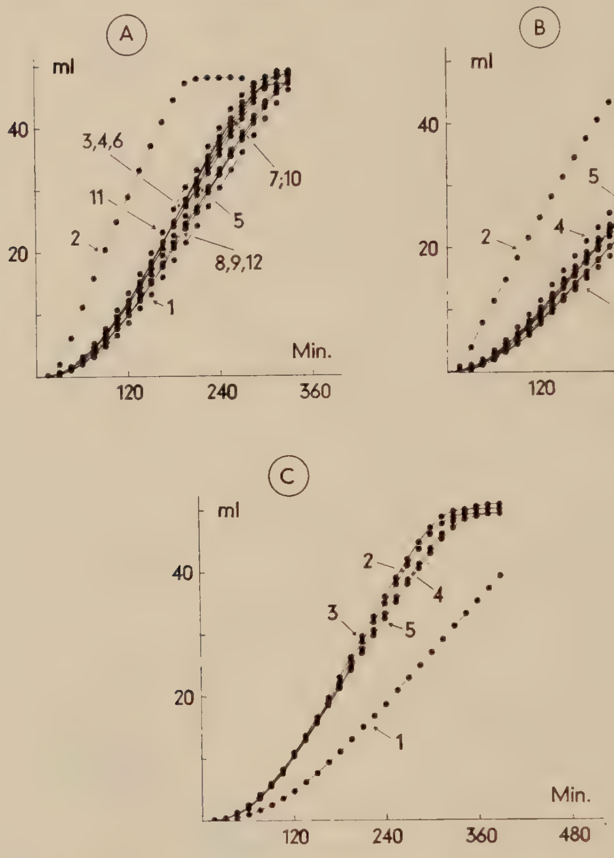


Abb. 3. Aufhebung der unter anaeroben Bedingungen in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer auftretenden Hemmung der Vergärung der Glucose durch Zuckerarten, Zuckeralkohole, Zuckerkarbonsäuren und Glucoside, welche den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff, d.h. aerob, beigemischt werden.

A: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Glucose,
3. Sorbit,
4. Mannit,
5. Dulcit,
6. Adonit,
7. Arabit,
8. Erythrit,
9. Glycerin,
10. α-Methyl-D-Glucosid,
11. α-Methyl-D-Mannosid,
12. β-Methyl-D-Xylosid.

B: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Glucose,
3. Gluconsäure-lacton,
4. Glucoheptonsaures Calcium,
5. Arabonsaures Calcium,
6. Glucoheptonsäure-lacton,
7. Arabonsäure-lacton,
8. Ribonsäure-lacton,
9. Galactonsäure-lacton,
10. Gulonsäure-lacton,
11. Galacturonsäure,
12. Glucuronsäure-lacton.

C: 1. Kein aerober Zusatz.
Aerober Zusatz von:
2. Galactose Mann Research Laboratories,
3. Galactose Hoffmann-La Roche,
4. D(—)-Ribose Mann Research Laboratories,
5. D(—)-Ribose Hoffmann-La Roche.

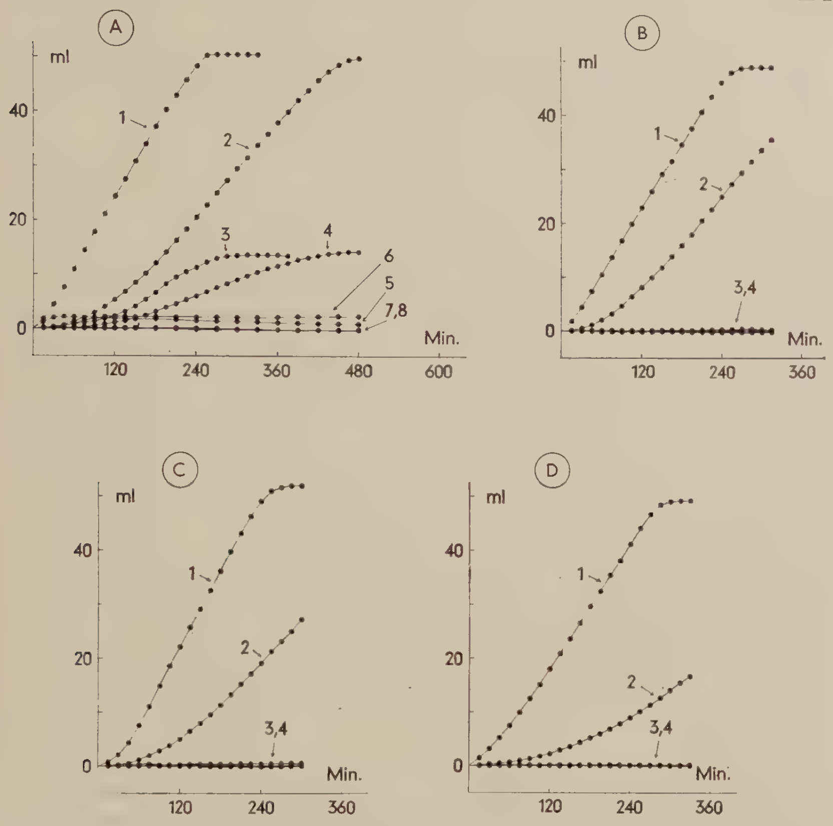
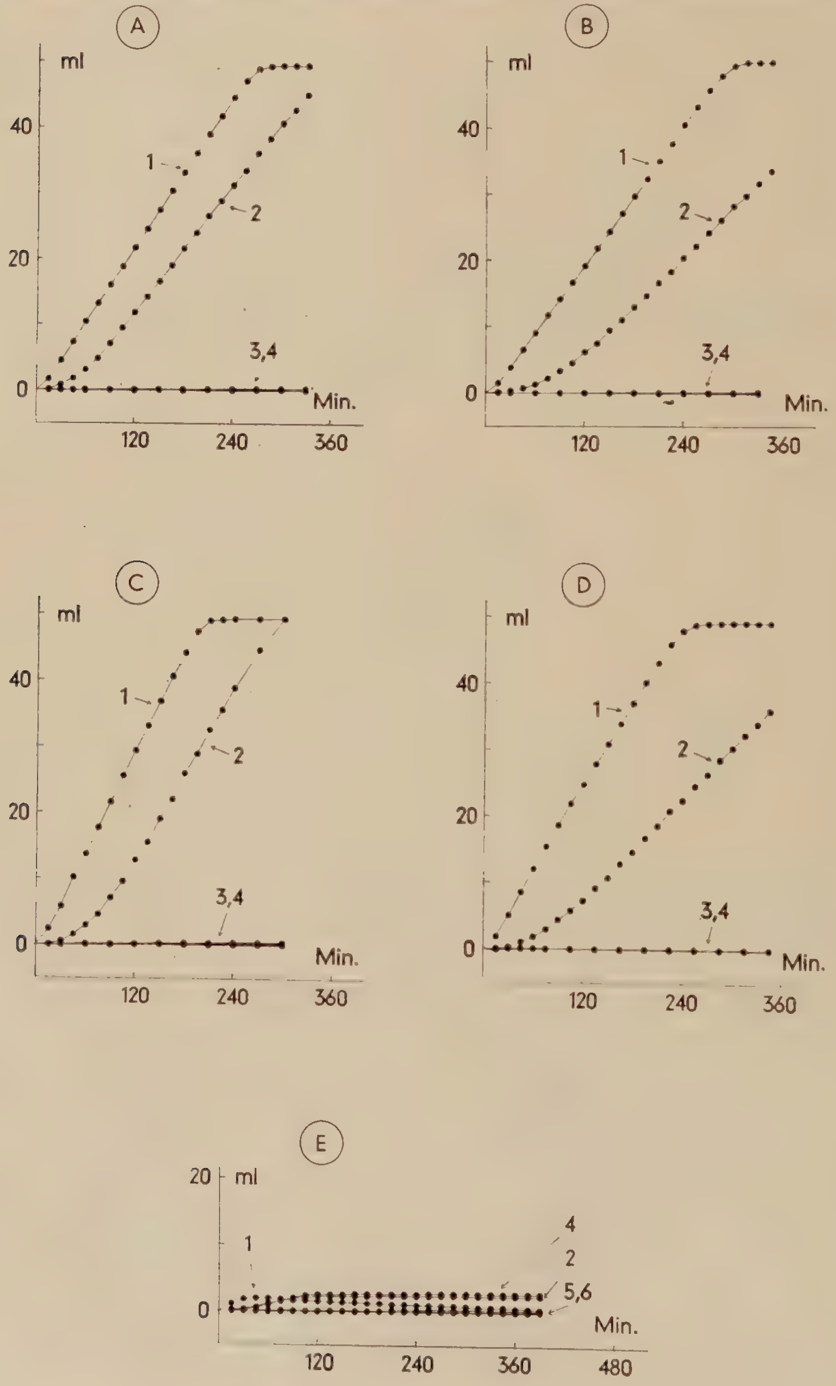


Abb. 4. Aerobe und anaerobe Vergärung verschiedener Zuckerarten und Polysaccharide in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer durch ruhende Zellen aus jungen Kulturen der Weinhefe „Fendant".

		Aerob	Anaerob
A:	Glucose	1	2
	Raffinose	3	4
	Galactose Hoffmann-La Roche	5	6
	Sorbose Rhamnose Fucose	7	8
B:	Fructose	1	2
	D(+)-Xylose L(—)-Xylose D(—)-Arabinose L(+)-Arabinose D(—)-Ribose Hoffmann-La Roche	3	4
C:	Saccharose	1	2
	Maltose Lactose Cellobiose Trehalose Melibiose	3	4
D:	Mannose	1	2
	Melezitose Turanose Glykogen α-Dextrin β-Dextrin	3	4


A
ml
40
20
0
1
2
3,4
120
240
360
Min.
B
ml
40
20
0
1
2
3,4
120
240
360
Min.
C
ml
40
20
0
1
2
3,4
120
240
360
Min.
D
ml
40
20
0
1
2
3,4
120
240
360
Min.
E
20
ml
0
1
4
2
5,6
120
240
360
480
Min.

Calcium-Salz eine äusserst geringe Hemmungsaufhebung, während D-Arabonsäure als Calcium-Salz sowie die Lactone der D-Gluconsäure und α-Glucoheptonsäure vollständig inaktiv sind und die Lactone der D-Arabonsäure, D-Ribonsäure, D-Galactonsäure und Gulonsäure eine zusätzliche Hemmung der anaeroben Vergärung der D-Glucose hervorrufen (je 1 Versuch).

Die D-Glucuronsäure als Lacton erwies sich gleich wie D-Galacturonsäure als völlig inaktiv (je 1 Versuch).

Die geprüften Glucoside zeigten abnehmende Hemmungsaufhebung in der Reihenfolge α-Methyl-D-Mannosid, α-Methyl-D-Glucosid und β-Methyl-D-Xylosid (je 1 Versuch), wobei die Wirkung durchgehend nur gering ist. Die letzte Verbindung kann sogar als praktisch inaktiv betrachtet werden.

Es sei noch erwähnt, dass das Gemisch der λ- und δ-Lactone der D-Gluconsäure in zahlreichen weiteren Versuchen bei verschiedenen Konzentrationen auf hemmungsaufhebende Wirkung geprüft wurde. Es erwies sich durchgehend als vollständig inaktiv.

In neun weiteren Versuchen wurde sodann die Vergärbarkeit der erwähnten Kohlehydrate, Zuckeralkohole, Zuckerkarbonsäuren und Glucoside unter aeroben und anaeroben Bedingungen geprüft. Die Ergebnisse sind aus Abb. 4 A - 4 D und 5 A - 5 E ersichtlich.

Abb. 5. Aerobe und anaerobe Vergärung verschiedener Zuckerarten, Zuckeralkohole, Zuckerkarbonsäuren und Glucoside in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer durch ruhende Zellen aus jungen Kulturen der Weinhefe „Fendant".

		Aerob	Anaerob
A:	Glucose	1	2
	Sorbit	3	4
	Mannit		
	Dulcit		
	Adonit		
	Arabit		
B:	Glucose	1	2
	Erythrit	3	4
	Glycerin		
	α-Methyl-D-Mannosid		
	α-Methyl-D-Glucosid		
	β-Methyl-D-Xylosid		
C:	Glucose	1	2
	Gluconsäure-lacton	3	4
	Glucoheptonsaures Calcium		
	Arabonsaures Calcium		
	Galacturonsäure		
	Glucuronsäure-lacton		
D:	Glucose	1	2
	Glucoheptonsäure-lacton	3	4
	Arabonsäure-lacton		
	Ribonsäure-lacton		
	Galactonsäure-lacton		
	Gulonsäure-lacton		
E:	Galactose Mann Research Laboratories	1	2
	Galactose Hoffmann-La Roche		4
	D(—)-Ribose Mann Research Laboratories	5	6
	D(—)-Ribose Hoffmann-La Roche		

TABELLE 1.

Auf Vergärbarkeit geprüfte Verbindung	Maximale Volumenzunahme (+) bzw. -abnahme (—) in ml	
	Anaerob	Aerob
D-Arabinose (Abb. 4 B)	0,0	+0,1
L-Arabinose (Abb. 4 B)	0,0	+0,1
D-Xylose (Abb. 4 B)	0,0	+0,3
L-Xylose (Abb. 4 B)	0,0	—0,2
D-Ribose Hoffmann-La Roche (Abb. 4 B bzw. 5 E)	+0,1 bzw. +0,3	0,0 bzw. +0,1
D-Ribose Mann Research Laboratories (Abb. 5 E)	+0,2	+0,2
L-Rhamnose (Abb. 4 A)	—0,2	+0,1 → —0,1
L-Fucose (Abb. 4 A)	—0,1	+0,1 → —0,2
L-Sorbose (Abb. 4 A)	—0,2	+0,2 → —0,1
Maltose (Abb. 4 C)	+0,6	+0,4 → —0,1
Trehalose (Abb. 4 C)	+0,1	+0,1 → 0,0
Cellobiose (Abb. 4 C)	+0,5	—0,1
Turanose (Abb. 4 D)	+0,1	+0,1
Lactose (Abb. 4 C)	0,0	0,0
Melibiose (Abb. 4 C)	+0,1	0,0
Melezitose (Abb. 4 D)	+0,1	+0,2
Glykogen (Abb. 4 D)	+0,2	0,0
α-Dextrin (Abb. 4 D)	+0,1	0,0
β-Dextrin (Abb. 4 D)	+0,1	+0,2
Glycerin (Abb. 5 B)	0,0	+0,1
Erythrit (Abb. 5 B)	0,0	+0,1
L-Arabit (Abb. 5 A)	+0,2	+0,2
Adonit (Abb. 5 A)	+0,1	+0,2
Dulcit (Abb. 5 A)	+0,2	+0,2
D-Mannit (Abb. 5 A)	+0,2	+0,3
D-Sorbit (Abb. 5 A)	+0,2	+0,2
D-Arabonsäure- γ -lacton (Abb. 5 D)	0,0	+0,1
D-Arabonsaures Calcium (Abb. 5 C)	+0,3	+0,3
D-Ribonsäure- γ -lacton (Abb. 5 D)	0,0	+0,1
D-Gluconsäure- γ -und- δ-lacton (Abb. 5 C)	+0,2	+0,3
D-Galactonsäure- γ -lacton (Abb. 5 D)	0,0	+0,1
Gulonsäure-lacton (Abb. 5 D)	+0,1	+0,1
α-Glucoheptonsäure-lacton (Abb. 5 D)	0,0	+0,1
Glucoheptonsaures Calcium (Abb. 5 C)	+0,1	+0,3
D-Glucuronsäure- γ -lacton (Abb. 5 C)	+0,2	+0,2
D-Galacturonsäure (Abb. 5 C)	+0,2	+0,3
β-Methyl-D-Xylosid (Abb. 5 B)	0,0	0,0
α-Methyl-D-Glucosid (Abb. 5 B)	+0,1	+0,1
α-Methyl-D-Mannosid (Abb. 5 B)	+0,1	+0,1

Man sieht, dass D-Glucose, D-Fructose, D-Mannose und Saccharose von den Zellen der ,,Fendant''-Hefe aus jungen Kulturen sowohl unter aeroben als auch anaeroben Bedingungen vergoren werden, wobei in sämtlichen Fällen die anaerobe Gärungshemmung deutlich zum Vorschein kommt. Die nach Vergärung von 200 mg D-Glucose in Luft eingetretene Volumenvergrösserung betrug bei beendeter Gärung 50,7 bzw. 49,2, 49,7, 49,2 und 49,2 ml (Abb. 4 A bzw. 5 A — 5 D), d. h. durchschnittlich 49,6 ml ($\delta = 0{,}5$). Für D-Fructose bzw. D-Mannose und Saccharose waren die entsprechenden Werte 49,0 bzw. 49,2 und 51,8 ml (Abb. 4 B bzw. 4 D und 4 C).

Es geht ferner hervor, dass auch Raffinose sowohl aerob als auch anaerob vergoren wird, wobei gleichfalls eine Hemmung der anaeroben Gärung auftritt. Es entwickelten sich in O_2-freiem Stickstoff maximal 14,3 ml Kohlendioxyd, während in Luft eine Volumenzunahme von 13,7 ml festgestellt werden konnte. Dies bedeutet, dass nur der D-Fructose-Teil des Trisaccharids Raffinose durch die ,,Fendant''-Zellen aus jungen Kulturen dissimiliert wird, während der Melibiose-Teil intakt bleibt. Wie wir später sehen werden, kann das Disaccharid Melibiose weder unter anaeroben, noch unter aeroben Bedingungen durch die Heferasse ,,Fendant'' vergoren werden.

Die beiden D-Galactose-Präparate werden in geringem Ausmasse vergoren. Unter anaeroben Bedingungen entwickelten sich im Falle des von der Firma Hoffmann-La Roche erhaltenen Präparats 2,3 bzw. 2,7 ml Kohlendioxyd (Abb. 4 A bzw. 5 E), während im Falle des von Mann Research Laboratories bezogenen Präparats (,,assayed'') maximal 2,5 ml gebildet wurden. Die in Luft gemessene Volumenvergrösserung betrug für das erste Präparat maximal 2,1 ml (Abb. 4 A) und für das letzte Präparat 1,9 ml (Abb. 5 E).

Die übrigen Zuckerarten können gleich wie die geprüften Polysaccharide, Zuckeralkohole, Zuckerkarbonsäuren und Glucoside als nicht vergärbar bezeichnet werden. Die maximalen Volumenveränderungen in ml sind in Tabelle 1 zusammengestellt (Zunahme: +; Abnahme: —). Aus diesen Daten ist klar ersichtlich, dass praktisch nur die Präparate von Maltose und Cellobiose eine gewisse, äusserst unbedeutende Kohlendioxydentwicklung ermöglichen (0,6 bzw. 0,5 ml nach 300 Min. in O_2-freiem Stickstoff).

Es wurden sodann fünf Versuche durchgeführt über die Abhängigkeit der Aufhebung der anaeroben Gärungshemmung von der

Zuckermenge, welche den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff aerob beigemischt wird. Die Ergebnisse dieser Experimente, in welchen durchgehend D-Glucose zur Verwendung kam, liegen in Abb. 6 A - 6 D und 7 vor.

Die in Abb. 6 A dargestellten Resultate zeigen, dass durch aeroben Zusatz von 1,0 mg D-Glucose eine deutliche partielle Beseitigung der anaeroben Gärungshemmung hervorgerufen wird, während im Konzentrationsbereich von 0,0001 bis 0,01 mg dieser Zuckerart keine aufhebende Wirkung nachgewiesen werden kann. Nach aerobem Beimischen von 100,0 mg D-Glucose verläuft die anaerobe Gärung mit bedeutend grösserer Geschwindigkeit als die aerobe Gärung.

Aus den in Abb. 6 B vorliegenden Gärkurven geht hervor, dass nach aerobem Zusatz von 0,02 bis 0,05 mg D-Glucose eine sehr kleine Stimulierung der anaeroben Gärung einsetzt, während durch aerobes Beimischen von 0,10 bzw. 0,50 mg dieses Zuckers eine deutliche bzw. bedeutende Aufhebung der anaeroben Gärungshemmung bewirkt wird.

Abb. 6 C zeigt, dass aerob zugesetzte D-Glucose in Mengen von 10,0 bzw. 50,0 und 100,0 mg die anaerobe Gärungshemmung vollständig beseitigt bzw. die anaerobe Gärung so stark stimuliert, dass sie mit wesentlich grösserer Geschwindigkeit verläuft als die aerobe Gärung ohne extra Zuckerzusatz.

Die in Abb. 6 D dargestellten Gärkurven bestätigen schliesslich, dass nach aerobem Zusatz von 0,05 bzw. 0,10 bis 100,0 mg D-Glucose eine nur geringe bzw. eine deutliche partielle bis vollständige Aufhebung der anaeroben Gärungshemmung hervorgerufen wird, wobei die Proportionalität zwischen dem Ausmasse der Hemmungsbeseitigung und der aerob beigemischten Zuckermenge sehr schön zum Vorschein kommt.

Die Kohlendioxydmengen, welche in den in Abb. 6 und 7 (vgl. unten) dargestellten Versuchen bei beendeter Gärung entwickelt worden waren, sind in Tabelle 2 zusammengestellt. Es ist ersichtlich, dass die kleineren D-Glucosemengen, welche den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff oder vor deren Schütteln in ruhender Luft beigemischt wurden, bereits vollständig vergoren sind, bevor die anaerob oder aerob zu vergärende Zuckermenge (200 mg) eingekippt wird. Nach aerobem Zusatz grösserer Mengen D-Glucose wurde in gewissen Versuchen nach beendeter Vergärung von 200 mg Zucker ein um etwa 1 ml

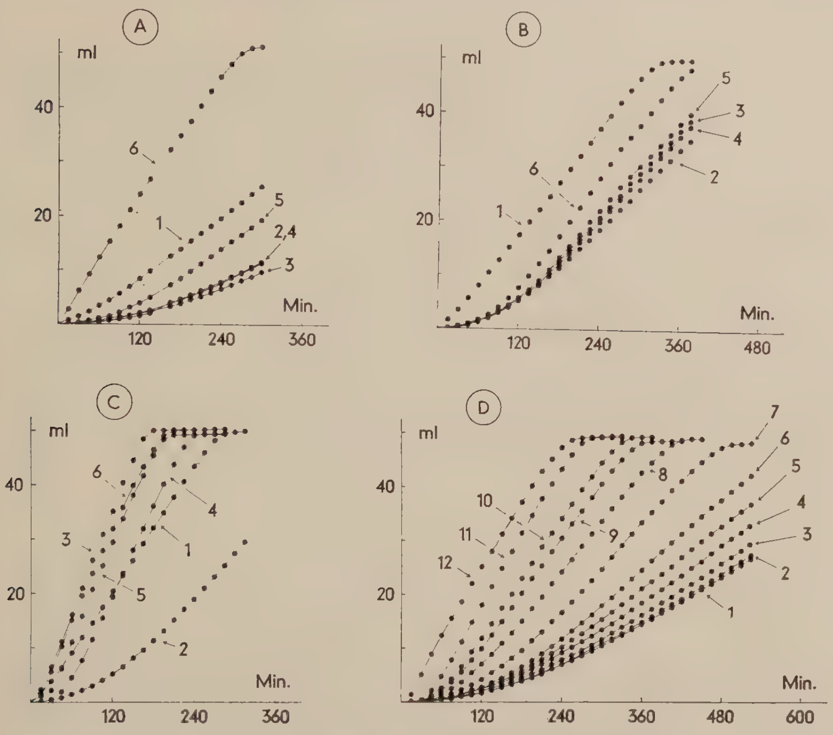


Abb. 6. Die Konzentrationsabhängigkeit der Aufhebung der anaeroben Hemmung der Glucosevergärung in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer durch Glucose, welche den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff, d.h. aerob, beigemischt wird.

		Aerob	Anaerob
A:	Kein aerober Zusatz	1	2
	Aerober Zusatz von:		
	0,0001 mg Glucose		3
	0,01 mg Glucose		4
	1,0 mg Glucose		5
	100,0 mg Glucose		6
B:	Kein aerober Zusatz	1	2
	Aerober Zusatz von:		
	0,02 mg Glucose		3
	0,05 mg Glucose		4
	0,10 mg Glucose		5
	0,50 mg Glucose		6
C:	Kein aerober Zusatz	1	2
	Aerober Zusatz von:		
	10,0 mg Glucose	3	4
	50,0 mg Glucose		5
	100,0 mg Glucose		6
D:	Kein aerober Zusatz		1
	Aerober Zusatz von:		
	0,05 mg Glucose		2
	0,10 mg Glucose		3
	0,25 mg Glucose		4
	0,50 mg Glucose		5
	1,0 mg Glucose		6
	2,5 mg Glucose		7
	5,0 mg Glucose		8
	7,5 mg Glucose		9
	10,0 mg Glucose		10
	25,0 mg Glucose		11
	100,0 mg Glucose		12

TABELLE 2.

Abb.	Vor der Begasung aerob zugesetzte Glucosemenge in mg	Gesamtvolumen des bei beendeter Gärung entwickelten Kohlendioxyds in ml	
		Anaerob	Aerob
6 A	100,0	51,5	—
6 B	0,0	—	50,3
6 C	0,0	—	49,9
	10,0	49,5	50,2
	50,0	49,4	—
	100,0	50,3	—
6 D	2,5	48,2	—
	5,0	48,9	—
	7,5	48,6	—
	10,0	49,3	—
	25,0	49,2	—
	100,0	49,4	—
7	0,5	49,6	—

vergrössertes Kohlendioxydvolumen erhalten. Dies entspricht unter den von uns gewählten experimentellen Bedingungen ca. 4 mg D-Glucose. In diesen Fällen waren also im Augenblick des Einkippens von 200 mg Zucker höchstens 4 - 8% der D-Glucosemengen unvergoren, welche den Zellsuspensionen vor deren Begasung mit O_2-freiem Stickstoff oder Luft beigemischt worden waren.

Wie oben erwähnt, konnte schon nach aerobem Zusatz von 0,10 mg D-Glucose eine deutliche partielle Aufhebung der anaeroben Gärungshemmung festgestellt werden. Da in den betreffenden Experimenten 300 mg Nasshefe, entsprechend ca. 60 mg Hefetrockensubstanz (55), zur Verwendung kamen, bedeutet dies, dass bereits 0,33 μg D-Glucose pro mg Nasshefe bzw. 1,67 μg Zucker pro mg Hefetrockensubstanz wirksam sind. Es liegt hier somit die Möglichkeit vor, eine biologische Methode zu entwickeln, mit deren Hilfe sehr kleine Zuckermengen qualitativ nachgewiesen bzw. quantitativ bestimmt werden können.

Im fünften Versuch über die hemmungsaufhebende Wirkung aerob zugesetzter D-Glucose kamen verschiedene Hefemengen, und zwar 75 bzw. 150 und 300 mg Nasshefe, sowie verschiedene Glucosemengen, und zwar 0,125 bzw. 0,25 und 0,50 mg, zur Verwendung. Die Ergebnisse liegen in Abb. 7 vor. Es ist ersichtlich, dass die anaerobe Gärungshemmung in sämtlichen Fällen nach aerobem Zuckerzusatz in beträchtlichem Ausmasse aufgehoben wurde. Für

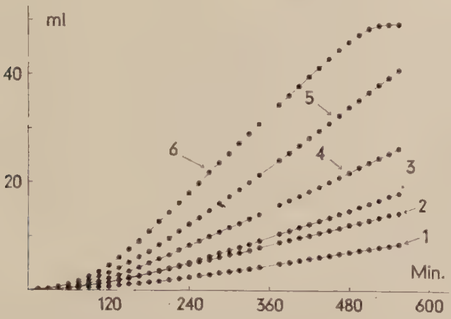


Abb. 7. Aufhebung der anaeroben Hemmung der Glucosevergärung in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer durch verschieden grosse Glucosemengen, welche Hefesuspensionen verschiedener Dichte vor deren Begasung mit O_2-freiem Stickstoff, d.h. aerob, beigemischt werden.

		Anaerob
75 mg Hefe	Kein aerober Zusatz	1
	Aerober Zusatz von 0,125 mg Glucose	2
150 mg Hefe	Kein aerober Zusatz	3
	Aerober Zusatz von 0,25 mg Glucose	4
300 mg Hefe	Kein aerober Zusatz	5
	Aerober Zusatz von 0,50 mg Glucose	6

die Zeitspanne 180 - 555 Min., innerhalb welcher die Gärkurven als approximativ linear betrachtet werden können, beträgt die Gärgeschwindigkeit bei den Hefesuspensionen ohne extra D-Glucose 0,285 bzw. 0,598 und 1,346 ml Kohlendioxyd pro 15 Min., während nach aerobem Beimischen von Zucker in der gleichen Zeitspanne bzw. im Zeitintervall 180 - 435 Min. (300 mg Hefe) 0,452 bzw. 0,856 und 1,794 ml pro 15 Min. erhalten wurden. Die ersten Gärgeschwindigkeiten verhalten sich wie 1,00 : 2,10 : 4,72 und die letzten wie 1,00 : 1,89 : 3,97. Diese Daten zeigen, dass die anaerobe Vergärung der D-Glucose (200 mg) durch die in O_2-freiem Stickstoff geschüttelten Hefesuspensionen erst nach aerobem Zusatz von Zucker mit Geschwindigkeiten verläuft, welche den betreffenden Zellmengen (1,00 : 2,00 : 4,00) proportional sind. Durch die zur Sicherung der Anaerobiose dienende Vorbehandlung in O_2-freiem Stickstoff scheint somit das Zymasesystem der verdünnteren Hefesuspensionen in verhältnismässig grösserem Ausmasse reversibel (vgl. S. 193–194) inaktiviert zu werden als dasjenige der konzentrierteren Suspensionen. Durch aerobes Beimischen von D-Glucose lässt sich die Proportionalität zwischen der Gärgeschwindigkeit in der annähernd geradlinigen Gärphase und der Hefemenge wiederherstellen.

Es schien uns schliesslich wichtig festzustellen, ob D-Glucose die anaerobe Gärungshemmung aufzuheben vermag, wenn sie den Hefe-

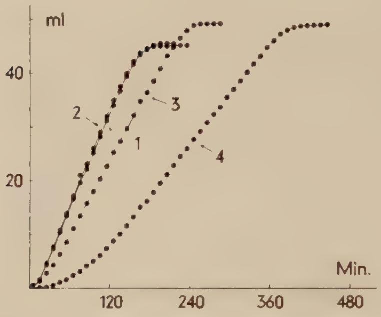


Abb. 8. Aufhebung der anaeroben Gärungshemmung in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer durch Glucose, welche bei 0,1° C. den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff, d.h. aerob, beigemischt wird.

	Aerob	Anaerob
Aerober Zusatz von Glucose bei 0,1° C.	1	2
Kein aerober Zusatz von Glucose vor dem Schütteln in O_2-freiem Stickstoff oder Luft	3	4

suspensionen aerob bei niedriger Temperatur beigemischt wird. Abb. 8 zeigt die Ergebnisse eines Versuches, in welchem die zu vergärende Zuckermenge (200 mg) einerseits vor dem Schütteln der Suspensionen in O_2-freiem Stickstoff oder Luft bei 0,1° C., anderseits, wie üblich, nach dem Schütteln bei 25° C. eingekippt wurde. Es ist ersichtlich, dass die bei niedriger Temperatur aerob zugesetzte Glucose ebenfalls zur vollständigen Beseitigung der anaeroben Gärungshemmung fähig ist. Ferner geht aus Abb. 8 hervor, dass die Hefe imstande ist, die Glucose bei 0,1° C. mit messbarer Geschwindigkeit zu vergären. Das Zeitintervall zwischen dem Zusetzen des Zuckers und dem Beginn des Schüttelns der betreffenden Hefesuspensionen in Luft bzw. O_2-freiem Stickstoff betrug 35 Min. und die Dauer der letzten Behandlung, wie bereits erwähnt, 120 Min. Während der gesamten Zeitspanne von 155 Min. entwickelten sich in beiden Fällen 3,6 ml Kohlendioxyd. Dies entspricht einer Gärgeschwindigkeit von ca. 0,35 ml Kohlendioxyd pro 15 Min. und 300 mg Nasshefe.

DISKUSSION UND SCHLUSSFOLGERUNGEN.

Wie bereits erwähnt, werden unter den gewählten experimentellen Bedingungen D-Glucose, D-Fructose, D-Mannose und Saccharose sowohl aerob als anaerob vollständig dissimiliert, wobei 49,5 (aerob bzw. anaerob durchschnittlich 49,8 und 49,2 ml) bzw.

49,0, 49,2 und 51,8 (aerob) ml Kohlendioxyd je 200 mg Zucker gebildet werden.

Bei der Dissimilation der Raffinose betrug die anaerobe bzw. aerobe Volumenvergrösserung 14,3 und 13,7 ml. Nun entsprechen 200 mg des verwendeten Raffinose-Präparats ($C_{18}H_{32}O_{16} + 5\ H_2O$, Hoffmann-La Roche) 60,6 mg freier D-Fructose. Das bei der Vergärung dieser Zuckermenge sich entwickelnde Kohlendioxydvolumen würde unter den experimentellen Bedingungen 14,8 ml betragen. Raffinose wird mithin nur zu einem Drittel, entsprechend dem Fructoseteil, dissimiliert. Dies ist im Einklang mit der Tatsache, dass Saccharose vergärbar ist, während Melibiose sich als nicht vergärbar erwiesen hat.

Bei der Prüfung der D-Galactose-Präparate (Hoffmann-La Roche bzw. Mann Research Laboratories „assayed") erhielten wir unter anaeroben bzw. aeroben Bedingungen maximal durchschnittlich 2,5 und 2,0 ml Kohlendioxyd je 200 mg Zucker. Bekanntlich wird D-Galactose meistens durch saure Hydrolyse der Lactose und anschliessende fraktionierte Kristallisation des entstehenden Galactose-Glucose-Gemisches oder Vergärung des letzten Zuckers mit einer geeigneten Hefe hergestellt (3, 4, 17, 33, 38, 39, 42, 46). Es ist deshalb wahrscheinlich, dass die geprüften D-Galactose-Präparate durch D-Glucose verunreinigt sind (Lactose ist unvergärbar). Nun entsprechen unter den gewählten experimentellen Bedingungen 2,5 ml Kohlendioxyd 10,1 mg D-Glucose. Die D-Galactose-Präparate enthalten mithin höchstens 5 Gewichts-% einer vergärbaren Zuckerart.

In den Maltose- bzw. Cellobiose-Lösungen entwickelten sich unter anaeroben Bedingungen maximal 0,6 und 0,5 ml Kohlendioxyd pro 200 mg Zucker. Dies deutet darauf hin, dass die erwähnten Disaccharide durch etwa 1 Gewichts-% eines vergärbaren Zuckers verunreinigt sind.

Für die übrigen auf Vergärbarkeit geprüften Präparate konnte unter anaeroben und aeroben Bedingungen gar keine oder höchstens eine minimale Kohlendioxydbildung festgestellt werden. Die im letzten Falle beobachteten Volumenvergrösserungen betrugen nur 0,1–0,3 ml und können nicht als gesichert betrachtet werden. Die betreffenden Substanzen sind demnach unvergärbar und enthalten keine vergärbaren Kohlehydrate oder höchstens Spuren ($\lesseqgtr$ 0,2–0,5%) solcher Verbindungen.

Aus den Ergebnissen der Vergärbarkeitsprüfung geht ferner eindeutig hervor, dass der Hemmungseffekt nicht auf die Vergärung der D-Glucose beschränkt ist, sondern bei sämtlichen vergärbaren Verbindungen auftritt, und zwar auch wenn diese Verbindungen, wie Raffinose, nur zum Teil vergoren werden oder nur als Verunreinigungen nicht vergärbarer Substanzen, wie in D-Galactose, vorliegen (D-Glucose: Abb. 4 A, 5 A - 5 D sowie 6 A - 6 C und 8; D-Fructose: Abb. 4 B; D-Mannose: Abb. 4 D; Saccharose: Abb. 4 C; Raffinose: Abb. 4 A; D-Galactose: Abb. 4 A und 5 D). Hinsichtlich des Gärsubstrats ist der Hemmungseffekt somit genereller Natur.

Gemäss LODDER und KREGER-VAN RIJ (25) sind Stämme der Art *Saccharomyces carlsbergensis* Hansen dadurch charakterisiert, dass sie zur vollständigen Vergärung der D-Glucose, D-Galactose, Saccharose, Maltose und Raffinose befähigt sind, während Lactose nicht angegriffen wird. Unter den von uns gewählten experimentellen Bedingungen erwies sich nicht nur Lactose, sondern auch D-Galactose und Maltose als unvergärbar. Ferner wird Raffinose nur zu einem Drittel, entsprechend dem Fructoseteil, vergoren. Diese Diskrepanzen zwischen unseren Ergebnissen und denjenigen von LODDER und KREGER-VAN RIJ (25) sind nicht erstaunlich in Anbetracht der Tatsache, dass die letzten Autoren bei der Vergärbarkeitsprüfung Lösungen der betreffenden Zuckerarten in Hefewasser in Gärkölbchen nach Einhorn verwendeten. Unter diesen Bedingungen, wo Wachstum, Vermehrung, Adaptation, Induktion, Mutation und Selektion und in Zusammenhang damit Änderungen in der enzymatischen Ausrüstung der Hefepopulation stattfinden können, gelang es auch WIKÉN und RICHARD (51), beim untersuchten Stamm von *Saccharomyces carlsbergensis* Hansen praktisch das gleiche Gärspektrum festzustellen wie LODDER und KREGER-VAN RIJ (25).

Wie bereits angeführt, heben D-Glucose, D-Fructose, D-Mannose und Saccharose die anaerobe Gärungshemmung auf, wenn sie den Hefesuspensionen vor deren Begasung mit O_2-freiem Stickstoff aerob beigemischt werden. Selbstverständlich kann diese Erscheinung auch als eine durch den aeroben Zusatz der erwähnten Zuckerarten hervorgerufene Verhinderung des Eintretens der Gärungshemmung beschrieben werden. Die Reaktivierung oder, wenn man so will, Stabilisierung des Gärvermögens der Hefezellen kommt teils in einem partiellen bis quantitativen Entfernen der Induktionsperiode, teils in einer Erhöhung der Geschwindigkeit der Kohlendioxydentwicklung in der approximativ linearen Gärphase zum Ausdruck. Die hemmungsaufhebende Wirkung kommt gerade zum

Vorschein nach Zusatz von 0,02 - 0,05 mg D-Glucose, während 0,10 - 1,0 mg dieses Zuckers eine bedeutende aber noch unvollständige und erst etwa 10,0 mg eine vollständige Beseitigung der Hemmung bewirken. Nach aerobem Zusatz von 50 - 100 mg D-Glucose konnte unter anaeroben Bedingungen ein Gärvermögen festgestellt werden, das viel grösser war als dasjenige, das für die parallelen aeroben Hefesuspensionen ohne extra Zuckerzusatz gemessen wurde. Unter den gewählten experimentellen Bedingungen (0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer, pH = 4,9; 300 mg frische Hefe, entsprechend etwa 60 mg Trockensubstanz; 120 Min. Begasung mit O_2-freiem Stickstoff) beträgt mithin die noch aktive Mindestmenge D-Glucose 1 - 3 μg pro 15 mg frische Hefe bzw. 3 mg Hefetrockensubstanz, entsprechend einem Mengenverhältnis von 1 - 3 : 15000 bzw. 1 - 3 : 3000. D-Fructose, D-Mannose und Saccharose sind allem Anschein nach etwa gleich aktiv wie D-Glucose. In sämtlichen Fällen handelt es sich um Zuckerarten, welche rasch und vollständig dissimiliert werden.

Die Abhängigkeit der Geschwindigkeit der anaeroben (und aeroben) Gärung in der approximativ linearen Phase von der Menge der aerob zugesetzten extra Glucose geht aus den in Tabelle 3 zusammengestellten Werten hervor. Die entsprechende Kurve für den in Abb. 6 D dargestellten Versuch liegt in Abb. 9 A vor. Die aus diesen Werten berechnete spezifische Wirkung des extra Zuckers, ausgedrückt als Gärgeschwindigkeitserhöhung in ml pro 1 mg Glucose, 300 mg feuchte Hefe und 15 Min., nimmt in sämtlichen Versuchen mit zunehmender Zuckermenge stark ab. Beispielsweise beträgt sie in dem erwähnten Versuch 1,10 bzw. 1,41, 0,95, 0,75, 0,57, 0,37, 0,24, 0,20, 0,17, 0,090 und 0,022 bei Zusatz von 0,05, 0,10, 0,25, 0,50, 1,0, 2,5, 5,0, 7,5, 10,0, 25,0 und 100,0 mg extra Glucose. Abb. 9 B zeigt die entsprechende Kurve.

Eine vollständige oder praktisch vollständige Aufhebung der Hemmung der anaeroben Glucosevergärung wird, wie bereits erwähnt, auch durch aeroben Zusatz von 50 mg Raffinose bewirkt. Diese Trisaccharidmenge entspricht 15,1 mg D-Fructose. Unter der Voraussetzung, dass die in Raffinose gebundene D-Fructose das gleiche aufhebende Vermögen wie freie D-Glucose besitzt, würde man eine vollständige Hemmungsbeseitigung erwarten, was ja tatsächlich auch zutrifft.

Auch D-Galactose und D-Ribose sind durch eine relativ starke hemmungsaufhebende Fähigkeit gekennzeichnet. Nun ging es aus

TABELLE 3.

Vor der Begasung aerob zugesetzte Glucosemenge in mg	Gärgeschwindigkeit in der annähernd geradlinigen Gärphase in ml Kohlendioxyd pro 300 mg feuchte Hefe und 15 Min.							
	Abb.							
	6 A		6 B		6 C		6 D	7
	Aerob	Anaerob	Aerob	Anaerob	Aerob	Anaerob	Anaerob	Anaerob
0,00	1,427	0,875	2,372	1,823	2,747	2,000	1,024	1,356
0,02	–	–	–	1,984	–	–	–	–
0,05	–	–	–	1,944	–	–	1,079	–
0,10	–	–	–	2,038	–	–	1,165	–
0,25	–	–	–	–	–	–	1,261	–
0,50	–	–	–	2,564	–	–	1,400	1,757
1,0	–	1,315	–	–	–	–	1,595	–
2,5	–	–	–	–	–	–	1,950	–
5,0	–	–	–	–	–	–	2,238	–
7,5	–	–	–	–	–	–	2,514	–
10,0	–	–	–	–	4,580	3,935	2,714	–
25,0	–	–	–	–	–	–	3,264	–
50,0	–	–	–	–	–	4,394	–	–
100,0	–	2,893	–	–	–	4,311	3,192	–

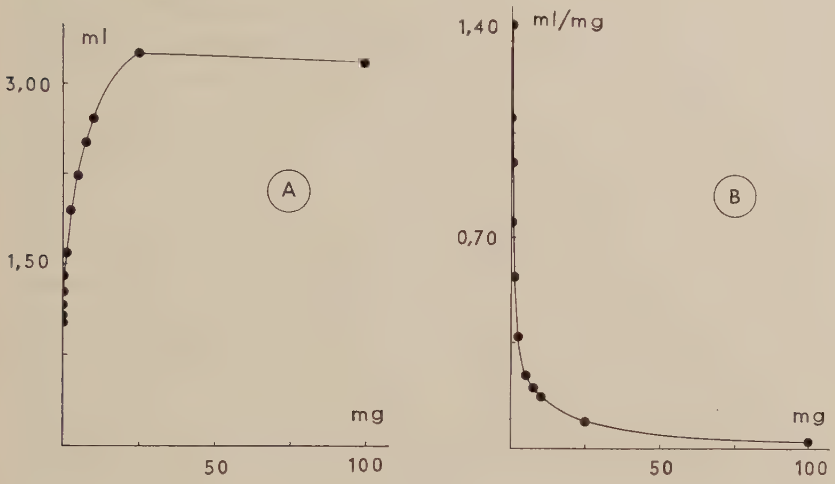


Abb. 9. Die Aufhebung der in 0,16 M Bernsteinsäure-Kaliumsuccinat-Puffer unter anaeroben Bedingungen auftretenden Hemmung der Vergärung der Glucose durch aerob zugesetzte Mengen dieses Zuckers (vgl. Abb. 6 D und Tabelle 3).

A: Die Abhängigkeit der absoluten Gärgeschwindigkeit in der annähernd linearen Phase, ausgedrückt in ml Kohlendioxyd je 300 mg feuchte Hefe und 15 Min., von der aerob zugesetzten extra Glucosemenge in mg.

B: Die Abhängigkeit der spezifischen Wirkung (Gärgeschwindigkeitserhöhung in ml/Menge extra Zucker in mg), ausgedrückt in ml pro 1 mg Zucker, 300 mg feuchte Hefe und 15 Min., von der aerob zugesetzten extra Glucosemenge in mg.

den Ergebnissen der Gärversuche hervor, dass die geprüften D-Galactose-Präparate durch höchstens 5 Gewichts-% eines vergärbaren Zuckers, wahrscheinlich D-Glucose, verunreinigt sind. Dies bedeutet, dass die betreffenden Hefesuspensionen nach aerobem Zusatz von 50 mg der D-Galactose-Präparate maximal 2,5 mg der vergärbaren Zuckerart enthalten. Gemäss den obigen Ausführungen ist diese Menge genügend, um eine bedeutende partielle Beseitigung der Hemmung der anaeroben Glucosevergärung hervorzurufen. Die von uns nach aerobem Zusatz von D-Galactose festgestellten Gärgeschwindigkeiten in der approximativ linearen Phase betrugen 3,875 (Abb. 1 A), 2,275 (Abb. 2 C) und 2,973 (Abb. 3 C) ml pro 300 mg feuchte Hefe und 15 Min., während für die Hefesuspensionen ohne solchen Zusatz 2,596, 1,467 und 1,993 ml Kohlendioxyd gemessen wurden. Die absoluten Gärgeschwindigkeitserhöhungen waren mithin 1,279 bzw. 0,808 und 0,980 ml. Unter der Voraussetzung, dass 50 mg der D-Galactose-Präparate 2,5 mg D-Glucose enthalten, werden für die spezifische Wirkung der Glucose Werte von 0,51 bzw. 0,32 und 0,39 erhalten gegenüber 0,37 ml pro mg extra Zucker in dem in Abb. 6 D dargestellten Versuch. Die Aktivität der an und für sich unvergärbaren D-Galactose lässt sich somit durch ihre Verunreinigung mit einem vergärbaren Zucker leicht erklären. In den mit 50 mg der D-Ribose-Präparate aerob versetzten Hefesuspensionen entwickelten sich in der annähernd linearen Gärphase 2,611 (Abb. 2 B) bzw. 2,150 (Abb. 2 D) und 2,733 (Abb. 3 C) ml Kohlendioxyd pro 300 mg frische Hefe und 15 Min., wobei die entsprechenden Suspensionen ohne extra Zuckerzusatz Gärgeschwindigkeiten von 1,872, 1,109 und 1,993 ml zeigten. Die absoluten Erhöhungen betragen mithin 0,739 bzw. 1,041 und 0,740 ml. Pro 200 mg der erwähnten Präparate werden durchschnittlich maximal nur 0,2 ml Kohlendioxyd gebildet. Dies entspricht höchstens 0,1 - 0,2 mg eines vergärbaren Zuckers pro 50 mg der Präparate, eine Quantität, die zwar genügt, um eine deutliche hemmungsaufhebende Wirkung hervorzurufen, aber viel zu klein ist, um das bedeutende Ausmass der experimentell festgestellten Hemmungsbeseitigung zu erklären. Es scheint mithin nicht unwahrscheinlich, dass D-Ribose, obgleich sie unter den gewählten experimentellen Bedingungen vom untersuchten Hefestamm nicht dissimiliert wird, doch durch eine verhältnismässig kräftige hemmungsaufhebende Aktivität gekennzeichnet ist.

Die mit 50 mg des Maltose-Präparats aerob versetzten Hefesuspensionen wiesen in der annähernd linearen Phase Gärgeschwindigkeiten von 2,800 (Abb. 1 B) und 1,864 (Abb. 2 C) ml Kohlendioxyd pro 300 mg feuchte Hefe und 15 Min. auf, während die entsprechenden Suspensionen ohne extra Zucker 2,336 bzw. 1,467 ml entwickelten. Dies entspricht absoluten Gärgeschwindigkeitserhöhungen von 0,464 und 0,397 ml und spezifischen Wirkungen von 0,93 bzw. 0,79 ml pro 1 mg des im geprüften Disaccharid-Präparat in einer Menge von höchstens etwa 1 Gewichts-% vorhandenen vergärbaren Zuckers. Zum Vergleich sei nochmals erwähnt, dass in dem in Abb. 6 D sowie 9 A und 9 B dargestellten Versuch beim aeroben Zusatz von 0,25 bzw. 0,50 mg D-Glucose spezifische Wirkungen von 0,95 und 0,75 ml gefunden wurden. Die hemmungsbeseitigende Wirkung der an und für sich nicht vergärbaren Maltose lässt sich mithin durch ihre Verunreinigung mit einer vergärbaren Zuckerart erklären. Das geprüfte Cellobiose-Präparat war in gleichem Ausmasse wie das Maltose-Präparat durch dissimilierbaren Zucker verunreinigt, vermag aber nur eine verhältnismässig kleine und ausserdem schlecht reproduzierbare Hemmungsaufhebung hervorzurufen. Für die Hefesuspensionen mit aerobem Zusatz von 50 mg dieses Disaccharids konnten Gärgeschwindigkeiten von 2,406 (Abb. 1 B) und 1,400 (Abb. 2 D) festgestellt werden. Die parallelen Suspensionen ohne solchen Zusatz entwickelten 2,336 bzw. 1,109 ml Kohlendioxyd pro 300 mg frische Hefe und 15 Min. Die absoluten Gärgeschwindigkeitserhöhungen waren also nur 0,070 bzw. 0,291 ml.

Wie bereits angeführt, können die übrigen geprüften Verbindungen als unvergärbar bezeichnet werden. Sie sind ferner gar nicht verunreinigt oder enthalten höchstens Spuren vergärbarer Kohlehydrate. In diesem Zusammenhang sei erwähnt, dass wir uns selbstverständlich dessen bewusst sind, dass in gewissen Fällen die Dissimilation der in einer unvergärbaren Verbindung vorliegenden sehr geringen Glucosemenge durch diese Verbindung dermassen gehemmt sein kann, dass wir sie unter den experimentellen Bedingungen nicht entdecken können. Einige dieser Verbindungen, wie die Lactone der D-Arabonsäure, D-Ribonsäure, D-Galactonsäure und Gulonsäure, bewirken eine kleine zusätzliche Gärungshemmung, während andere, wie D-Xylose, L-Sorbose, Lactose, Melibiose, D-Arabinose, Turanose, Melezitose, Glykogen, α-Dextrin, Inulin, L-Arabit, Dulcit, Glycerin, Erythrit, glucoheptonsaures und D-arabonsaures Calcium, D-Galacturonsäure, die Lactone der D-Gluconsäure, α-Glucoheptonsäure und D-Glucuronsäure sowie β-Methyl-D-Xylosid, völlig inaktiv sind oder höchstens eine äusserst geringe Hemmungsaufhebung hervorrufen. Die übrigen unvergärbaren Verbindungen, d. h. L-Arabinose, L-Rhamnose, L-Xylose, L-Fucose, Trehalose, β-Dextrin, Adonit, D-Mannit, D-Sorbit, α-Methyl-D-Mannosid und α-Methyl-D-Glucosid, bewirken eine etwas grössere aber immerhin verhältnismässig unbedeutende Hemmungsaufhebung.

Zusammenfassend kann gesagt werden, dass sämtliche vergärbare Kohlehydrate sowie die Präparate unvergärbarer Verbindungen, welche durch solche Kohlehydrate verunreinigt sind, zur Hemmungsaufhebung befähigt sind. Von den nicht dissimilierbaren Verbindungen ist eigentlich nur D-Ribose durch eine derart hohe hemmungsaufhebende Aktivität gekennzeichnet, dass diese nicht durch die Verunreinigung mit vergärbarem Kohlehydrat erklärt werden kann. Ferner sei bemerkt, dass in unseren Gärversuchen mit Präparaten unvergärbarer Substanzen, welche dissimilierbare Kohlehydrate als Verunreinigungen enthielten, weder unter anaeroben noch unter aeroben Bedingungen eine Synthese adaptiver oder induzierter Enzyme stattfand, welche eine Dissimilation der betreffenden unvergärbaren Substanzen hätte ermöglichen können.

Wir möchten schliesslich die von Lynen (26, 27), Lynen und Koenigsberger (28), Johnson (20), Chance, Connelly und Hess (9) sowie Chance und Hess (10) entwickelten Hypothesen über einen kausalen Zusammenhang zwischen den in den Zellen frei vorliegenden Mengen des anorganischen Phosphats bzw. der Adenosindiphosphorsäure und dem Pasteur-Meyerhof-Effekt in aller Kürze besprechen, und zwar im Lichte unserer eigenen Befunde und derjenigen von Stickland (44), Laws und Stickland (22), Aisenberg, Reinafarje und Potter (2) sowie Aisenberg und Potter (1).

Auf Grund der Arbeiten von Dische (14, 15), Needham und Pillai (34), Meyerhof und Mitarb. (30, 31, 32), Negelein und Brömel (35) sowie Warburg und Christian (47) wird bekanntlich angenommen, dass bei der Glykolyse und der alkoholischen Gärung die mit einer Reduktion der Cozymase gekoppelte Oxydation des 3-Phosphoglycerinaldehyds zu 3-Phosphoglycerinsäure von der Anwesenheit des anorganischen Phosphats und der Adenosindiphosphorsäure abhängig ist, indem gleichzeitig mit der erwähnten Oxydoreduktion aus den letzten beiden Verbindungen Adenosintriphosphorsäure synthetisiert wird. Dabei sollen 1,3-Diphosphoglycerinaldehyd und 1,3-Diphosphoglycerinsäure als Zwischenprodukte auftreten (vgl. Sumner und Somers (45), S. 345, Kaplan (21), S. 75). Lynen und Mitarb. (26, 27, 28) sowie Johnson (20) sind nun der Ansicht, dass der Pasteur-Meyerhof-Effekt darauf zurückzuführen sei, dass unter aeroben Bedingungen infolge der mit grosser Geschwindigkeit und in bedeutendem Ausmasse stattfindenden oxydativen Phosphorylierungen ein Mangel an freiem anorganischem Phosphat entstehe, wodurch die Umwandlung des Phosphoglycerinaldehyds in Phosphoglycerinsäure und sodann die ganze Glykolyse bzw. alkoholische Gärung gehemmt werden. Demgemäss sollten die betreffenden Zellen unter aeroben Bedingungen viel kleinere Mengen des anorganischen Phosphats enthalten als in Abwesenheit des molekularen Sauerstoffs. Diese Hypothese hat in einigen Fällen eine sehr beifällige Aufnahme gefunden (siehe z. B. Kaplan (21), S. 111, Hoffmann-Ostenhof (18), S. 707).

In dem stationären Zustand der Zellen, der dem dynamischen Gleichgewicht entspricht, das durch die Geschwindigkeiten der aeroben bzw. anaeroben Phosphorylierungs- und Dephosphorylierungsprozesse bedingt ist, haben nun Lynen und Mitarb. (26, 27,

28) sowie Stickland und Mitarb. (22, 44) tatsächlich einen kleinen Unterschied im Gehalt an freiem anorganischem Phosphat zwischen aerober und anaerober Hefe feststellen können. Wie Ochoa und Stern (37, S. 581) hervorheben, ist aber dieser Unterschied sehr wahrscheinlich zu klein, um allein die als Pasteur-Meyerhof-Effekt bezeichnete Hemmung der alkoholischen Gärung unter aeroben Bedingungen zu erklären.

Gemäss der von Chance und Mitarb. (9, 10) entwickelten Hypothese sei der Pasteur-Meyerhof-Effekt auf die Abnahme der Konzentration der freien Adenosindiphosphorsäure zurückzuführen, welche unter aeroben Bedingungen infolge der grossen Affinität dieser Substanz zu den in den Mitochondrien lokalisierten Bestandteilen der Atmungsketten stattfinden würde. Leider haben Laws und Stickland (22) keinen wesentlichen Unterschied in den Konzentrationen der Adenosinmono-, Adenosindi- und Adenosintriphosphate zwischen aerob (Sauerstoff) und anaerob (O_2-freier Stickstoff) dissimilierenden Hefezellen gefunden. Ferner konnten Aisenberg und Potter (1) feststellen, dass nach Zusatz von Mitochondrien zu einem von ihnen untersuchten glykolytischen System (ultracentrifugiertes Rattenhirn-Homogenat, Rattenleber-Mitochondrien) zwar ein Pasteur-Meyerhof-Effekt eintrat aber gleichzeitig praktisch keine Änderungen in den Konzentrationen der im Medium frei vorliegenden Adenosinphosphate (AMP, ADP und ATP) hervorgerufen wurden. Der Gehalt an diesen Verbindungen war sogar ein bisschen höher in dem mit Mitochondrien versetzten System als im parallelen System ohne solchen Zusatz.

Unabhängig davon, ob zwischen aerob dissimilierenden Zellen, welche eine Hemmung der Glykolyse bzw. der alkoholischen Gärung zeigen, und anaeroben Zellen Unterschiede im Gehalt an anorganischem Phosphat und Adenosindiphosphorsäure vorhanden sind oder nicht, sprechen unsere Versuchsergebnisse entschieden gegen das Vorhandensein eines Regulationsmechanismus des Pasteur-Meyerhof-Effekts in den von uns untersuchten Hefezellen im Sinne der von Lynen und Mitarb. (26, 27, 28), Johnson (20) sowie Chance und Mitarb. (9, 10) entwickelten Hypothesen, denn gerade unter aeroben Bedingungen (49, 50, 56, 57, 58, 59, 60, die vorliegende Arbeit) sowie unter anaeroben Bedingungen nach aerobem Zusatz von extra Zucker (50, die vorliegende Veröffentlichung), wo man einen zu niedrigen Gehalt an anorganischem Phosphat und Adenosindiphosphorsäure in den Hefezellen infolge reichlicher oxydativer Phosphorylierung erwarten würde, ist die alkoholische Gärung nicht durch eine Hemmung sondern durch eine Stimulierung gekennzeichnet. Diese nimmt im Bereich von 0,005 bis 10 à 20 Volumen-% Sauerstoff in der Gasphase (60) und von 0,05 bis 100,0 mg aerob beigemischter Glucose (50, die vorliegende Arbeit) mit steigender Konzentration des molekularen Sauerstoffs bzw. des Zuckers zu. Obgleich unsere Befunde sich mithin nicht durch die erwähnten Hypothesen erklären lassen, wollen wir selbstverständlich nicht sagen, dass die in diesen Hypothesen vorgeschlagenen Mechanismen nicht bei anderen Zellsystemen und unter anderen experimentellen Bedingungen spielen können.

Diese Untersuchungen wurden am Institut für landw. Bakteriologie und Gärungsbiologie, Eidg. Technische Hochschule, Zürich, ausgeführt und von der Abteilung für Landwirtschaft des Eidg. Volkswirtschaftsdepartements aus dem Weinbaufonds unterstützt. Dr. N. Pfennig's Studienaufenthalt am erwähnten Institut wurde durch ein Austauschstipendium des Deutschen Akademischen Austauschdienstes und der Eidg. Techn. Hochschule ermöglicht. Den zuständigen Behörden sprechen wir unseren herzlichen Dank aus. Fräulein A. Mohler danken wir bestens für sorgfältige Assistenz.

Die Artbestimmung der von Wikén und Richard (51) isolierten ,,Fendant''-Hefe (Stamm CBS 2834) verdanken wir Frau Dr. N. J. W. Kreger-van Rij, Hefeabteilung, Centraalbureau voor Schimmelcultures, Laboratorium voor Microbiologie, Delft.

Summary.

The present paper deals with the interaction of molecular oxygen, succinic acid-succinate and carbohydrates in limiting the rate of alcoholic fermentation in a strain of *Saccharomyces carlsbergensis* Hansen. This strain was isolated from a Fendant starter used for production of white wines of high alcoholic content at low temperatures (51). The Fendant strain is auxo-autotrophic but addition of (+)-biotin in amounts greater than 0.0025 - 0.025 μg 1000 ml of substrate and of meso-inositol in quantities greater than 0.25 - 2.5 mg/1000 ml results in a small increase in growth in synthetic nutrient solutions (51, 52, 61, 62).

The succinate effect described in this article was also found in other tame wine yeasts, e. g. in the Salenegg strain of *Saccharomyces uvarum* Beijerinck and in the Dézaley strain of *Saccharomyces cerevisiae* Hansen (59), and in brewery bottom yeasts, e. g. the Müllerbräu strain of *Saccharomyces cerevisiae* Hansen (58, 59). The Salenegg yeast is an auxo-autotrophic strain showing a marked growth

increase in synthetic media on addition of meso-inositol in amounts greater than 0.25 - 2.5 mg/1000 ml (52, 53, 61, 62) whereas the Dézaley and Müllerbräu yeasts are auxo-heterotrophic, the former strain requiring addition of a mixture of (+)-biotin, meso-inositol and D (+)-pantothenic acid or β-alanine for normal growth (48, 52, 54, 61, 62).

The Fendant yeast examined in the present work was grown for 15½ to 17½ hours at 20° C. or 23° C. in the vitamin-free medium C containing glucose, casein hydrolysate, potassium citrate buffer and mineral salts (51, 62). The carbon dioxide production was measured volumetrically at 25° C. using resting cells washed twice in distilled water and once in 0.16 M succinic acid-potassium succinate buffer at pH 4.9 and subsequently suspended in the buffer solution mentioned. As regards further details on general growth and fermentation conditions we refer to summaries given earlier (49, 55, 57, 59). In the aerobic fermentations the cell suspensions were shaken for 120 minutes in air and in the anaerobic fermentations for the same period of time in streaming oxygen-free nitrogen or for 100 minutes in streaming and subsequently for 20 minutes in resting nitrogen. After this pretreatment the compounds to be fermented aerobically (air) or anaerobically (oxygen-free nitrogen) were added in amounts of 50 mg (glycogen, α- and β-dextrin, neutralized lactones of D-gluconic, α-glucoheptonic, D-galactonic, gulonic, D-arabonic and D-ribonic acids) or in quantities of 200 mg (all other compounds tested) from the side bulb to the yeast suspension in the main compartment of the fermentation vessel. The substances examined for ability to counteract or reverse the inhibition of anaerobic and aerobic fermentation produced by succinic acid-succinate buffer were, if not stated otherwise, added aerobically in amounts of 50 mg previous to flushing with air or oxygen-free nitrogen.

On basis of the results of earlier investigations and of those described in the present paper the interaction of succinic acid-succinate, molecular oxygen and carbohydrates in limiting the rate of alcoholic fermentation in yeast may be characterized as follows:

In succinic acid-succinate buffer of pH 4.9 resting yeast cells from young (14 - 24 hours old) cultures show a strong inhibition of anaerobic alcoholic fermentation as compared to aerobic fermentation. This inhibition sets in after flushing with oxygen-free nitrogen or argon (49, 50, 56, 57, 58, 59, 60), hydrogen or carbon dioxide (49, 50). The rate of anaerobic carbon dioxide production decreases considerably with increasing flushing time (56, 57, 58, 59) and with increasing concentration of the succinic acid-succinate buffer (49, 50).

As far as we know, the inhibition mentioned is caused specifically by the succinic acid-succinate system. In the physiological concentration range at pH 4.9 acetic acid-acetate, citric acid-citrate, fumaric acid-fumarate and DL-malic acid-malate mixtures produce no inhibition and D-tartaric acid-tartrate only a minute inhibition of anaerobic carbon dioxide formation as compared to fermentation in air (49, 50).

At low concentrations succinic acid-succinate, citric acid-citrate, fumaric acid-fumarate, DL-malic acid-malate and D-tartaric acid-tartrate stimulate the fermentation in cells from young cultures, whereas in acetic acid-acetate an inhibiting effect upon the fermenting power as compared to that in distilled water is evident even at low concentrations (49).

The effect is observed in fermentation of all sugars dissimilated by the yeasts examined. Under the experimental conditions applied (0.16 M succinic acid-potassium succinate buffer of pH 4.9; 200 mg of sugar; 300 mg of wet yeast corresponding to about 60 mg of dry substance; shaking in air or oxygen-free nitrogen for 120 minutes previous to addition of the sugar to be fermented) only D-glucose, D-fructose, D-mannose and sucrose are being fermented completely and raffinose for one third (the fructose part of the molecule) by the Fendant strain of *Saccharomyces carlsbergensis* Hansen.

The strong inhibition of carbon dioxide formation produced by succinic acid-succinate buffer under anaerobic conditions is reversed by molecular oxygen supplemented to the gas phase of the fermenting systems previous to the addition of sugar to the yeast suspensions (56, 57, 58, 59). The fermentation rate increases with increasing oxygen tension in the gas phase in the concentration range of 0.005 to 10 or 20 per cent by volume (60). This means that even amounts of 2.5 to 3.0 μl of molecular oxygen are active per 300 mg of wet yeast or 60 mg of dry substance of yeast, corresponding on weight basis to ratios of approximately 1 - 1.5 : 100000 and 1 - 1.5 : 20000 respectively.

Further, the inhibition is counteracted by fermentable sugars, i. e. D-glucose, D-fructose, D-mannose, sucrose and raffinose, and by D-ribose added aerobically to the yeast suspensions previous to flushing with oxygen-free gas. This stimulation includes a shortening of the induction period of fermentation and an increase in rate of carbon dioxide production in the approximately linear phase of dissimilation. The fermentation capacity increases with increasing amount of extra sugar added in the range of 0.02 or 0.05 to 100 mg, the inhibition of anaerobic dissimilation as compared to that in air being completely removed under the experimental conditions applied on addition of about 10 mg of D-glucose. Thus, a stimulation is visible even on addition of only 1 to 3 μg of sugar per 15 mg of wet yeast or 3 mg of yeast dry substance, corresponding on weight basis to ratios of 1 - 3 : 15000 and 1 - 3 : 3000 respectively. The specific activity of the aerobically added D-glucose, calculated as increase in fermentation rate in the approximately linear phase in ml per 1 mg of extra sugar, decreases considerably with increasing amount of sugar.

The fact that a marked stimulation is caused by supplementing the yeast suspensions with molecular oxygen or extra sugar, as described above, speaks in favour of the improbability of the hypotheses proposed by LYNEN *et al.* (26, 27, 28), JOHNSON (20) and CHANCE *et al.* (9,10) to explain the mechanism of the PASTEUR-MEYERHOF effect on basis of the phosphate cycle as an inhibition of rate of glycolysis and alcoholic fermentation due to a decrease in the available amounts of inorganic phosphate and adenosine diphosphate which is supposed to set in on transferring the cells concerned from anaerobic to aerobic conditions. Under conditions which may be assumed to favour oxidative phosphorylation and thus cause a decrease in cellular contents of inorganic phosphate and adenosine diphosphate, i. e. in air or in synthetic mixtures of nitrogen and oxygen with increasing concentration of the latter gas and, further, in oxygen-free nitrogen after aerobic addition of increasing amounts of sugar serving as substrate for respiration and fermentation, we, as a matter of fact, do not find any inhibition of fermentation power but a stimulation of this power increasing with increasing tension of molecular oxygen in the atmosphere of the yeast suspensions and with increasing amounts of extra sugar added aerobically to these suspensions previous to flushing with oxygen-free gas. Although our results cannot be explained on basis of the hypotheses mentioned we, of course, do not deny that the mechanisms proposed may be of some significance in regulating the rate of fermentation in other cell systems and under other experimental conditions.

Non-fermentable carbohydrates (except D-ribose, as mentioned above), aldonic and uronic acids, polyhydric alcohols and glycosides are completely inactive or only slightly active in abolishing inhibition. In the cases where preparations of these compounds added aerobically showed ability to counteract the anaerobic inhibition of fermentation this could in general be explained as due to fermentable sugar present as impurity in the preparations concerned (D-galactose, maltose, cellobiose).

Cell material harvested from old cultures (63 - 72 hours of incubation at 20° C. or 23° C.) of the yeasts investigated, i.e. the Fendant strain of *Saccharomyces carlsbergensis* Hansen (CBS 2834), the Salenegg strain of *Saccharomyces uvarum* Beijerinck (CBS 2986), the Dézaley (CBS 2897) and Müllerbräu strains of *Saccharomyces cerevisiae* Hansen, shows none or only minute effects of the kind described (49, 56, 57, 58, 59).

The succinic acid-succinate effect is not related to any substance or substances exchangeable between yeast cells from young and old cultures in the same way as growth factors between auxo-autotrophic and auxo-heterotrophic micro-organisms in mixed cultures. The substance or substances involved in the inhibitions and stimulations connected with the succinic acid-succinate effect are strongly retained by the cellular structures or ar least incapable of penetrating the cytoplasmic membrane and wall of the yeast cells under the experimental conditions (59).

Further, the effect is an inherent physiological property characteristic of the yeast cells of generations developing during quite early phases of the growth cycle. It is a constituent part of the orderly modification which the yeast populations undergo with culture age irrespective of the changes in the chemical composition of the substrate taking place during growth and multiplication of the yeast (58, 59).

In succinic acid-succinate buffer of definite concentrations the yeast cells from young cultures are to be considered as microaerophilic to aerobic as regards the alcoholic fermentation. Cells from old yeast cultures are under the same conditions anaerobic as to this process in the sense that they ferment at a normal rate either in presence of only infinitesimal amounts of molecular oxygen or in complete absence of this gas in the environment.

Runnström *et al.* (40, 41) as well as Brandt (5, 6, 7) have postulated that succinic acid-succinate buffer is an indifferent system not poisoning yeast. The experimental results summarized above prove conclusively that this assumption is incorrect as far as yeast cells from young cultures are concerned. As a matter of fact, this buffer is not indifferent even in the case of cells from comparatively old cultures of the yeast strains investigated (69 hours of incubation at 20° C. or 23° C.), the fermenting power of such cells being stimulated at low and suppressed at high concentrations of succinic acid-succinate as compared to the fermenting capacity observed for the same cell material suspended in distilled water (49).

The stimulating effect produced by molecular oxygen in succinic acid-succinate solutions on the alcoholic fermentation in cells from young cultures of the strains of *Saccharomyces carlsbergensis* Hansen, *Saccharomyces cerevisiae* Hansen and *Saccharomyces uvarum* Beijerinck so far examined should be considered as a phenomenon different from the so-called negative PASTEUR effect discovered by CUSTERS (12) in a strain of *Brettanomyces claussenii* Custers and later found by WIKÉN *et al.* (63) in all *Brettanomyces* species tested. On the whole we, for the present, find it inappropriate to use the term ,,negative PASTEUR effect'' for one or other of these phenomena even though they have the property in common with the ,,normal PASTEUR effect'' that molecular oxygen somehow functions as a regulating agent on the rate of alcoholic fermentation. It seems to be wise to postpone the introduction of any terms giving the impression of a close relation of the different phenomena until not only the mechanism or mechanisms of the stimulating action of molecular oxygen but also that or those of the suppressing action of this gas on alcoholic fermentation and glycolysis are fairly well known.

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BACTERIOLOGICAL REQUIREMENTS FOR AND BACTERIOLOGICAL ANALYSIS OF PRECOOKED ('INSTANT') CEREALS AND SIMILAR FOODS

by

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Precooked ('instant') cereal products have been shown to contain occasionally pathogenic *Enterobacteriaceae* (19) and may, therefore, cause outbreaks of enterobacteriosis in infants (17). It has been demonstrated (6) that a non-specific sterility test, such as the EMMERLING mould test adopted in the Cereals Regulation under The Netherlands Food Law, gives inadequate protection against the potential health risk, involved in the use of such products.

Precooked cereals must be safe, especially for babies, when consumed without preliminary heating and also after storage, in reconstituted form, for some time at ambient temperatures. Therefore, they should be subjected to the same bacteriological requirements as applied currently to pasteurized foods such as bottled milk (4), ice cream (7), egg products (2), cooked sausages (11) and hot snacks (5). Such standards involve: absence of pathogenic *Enterobacteriaceae* in about 25 grammes of food and of all other *Enterobacteriaceae* at levels over 10 per one gramme of food; while the plate count should not exceed the order of 10^4 per gramme.

It seems desirable, also to limit the numbers of staphylococci, faecal streptococci, sulphite-reducing clostridia and *B. cereus* (3) to less than 10^2 per gramme (9), because as already mentioned reconstituted instant foods must be entirely safe for juvenile consumers also.

To discover, whether the manufacturers preparing such products could meet these requirements, 65 samples of instant cereals were bought in the open market and analysed bacteriologically. About twenty samples of other precooked foods, such as instant puddings and mixtures of dehydrated mashed potatoes and various vegetables, were analysed for comparative purposes.

MATERIALS ANALYSED.

The following dehydrated precooked foods, prepared in The Netherlands, were tested in this study:

(i) Instant baby cereals;
(ii) Precooked wheat;
(iii) Instant rolled oats;
(iv) Corn flakes;
(v) Puffed rice;
(vi) Instant puddings of various flavours;
(vii) Precooked dehydrated mashed potatoes and instant mixtures of vegetables and potatoes;
(viii) Soluble cocoa.

All popular brands were sampled and the number of samples taken of any commodity corresponded roughly with their sale in The Netherlands' market.

METHODS OF BACTERIOLOGICAL ANALYSIS.

In all samples the following groups of bacteria were counted.

(1) *Salmonella* (14).

About 25 grammes of the food were enriched in about 250 ml of plain tetrathionate broth (MULLER); tentative assays had shown that the products were virtually free from other *Enterobacteriaceae*, so that it was unnecessary to add further inhibitory agents to the tetrathionate medium. After 24 hours incubation at 37° C. subcultures were made onto brilliant green/phenol red/agar, desoxycholate/citrate/agar and SS-agar; after 17—24 hours incubation at 37° C. suspicious colonies were studied biochemically and serologically.

(2) Total *Enterobacteriaceae* (13).

One ml quantities of 1 : 10 dilutions of the products in 0.1% peptone/saline (14) were plated in duplicate in crystal violet/neutral red/bile/lactose/mannitol/agar and purple colonies were counted after 18 hours incubation at 37° C.

An adequate proportion of typical colonies was subcultured onto lactose/phenol red/agar and, after microscopic identification as pleomorphic Gram-negative rods, confirmed as *Enterobacteriaceae* by testing for fermentation of dextrose in the butt/slant-technique and for nitrate reduction (14).

(3) Faecal streptococci (12).

One ml quantities of 1 : 10 dilutions of the products were counted in tubes of Packer's crystal violet/sodium azide/blood/agar, incubated for 3 days at 39.5 ± 0.1° C.

Representative numbers of colonies were subcultured onto agar at 37° and 45 ± 0.1° C. The 45° C. negative cultures, which mostly appeared to be *Aerococcus* species (18), were discarded; while those positive at 45° C. when confirmed as catalase-negative streptococci, were tested for development in azide media (12) and, in case of doubt, also for the so-called SHERMAN characters of D-group streptococci (16).

(4) Sulphite reducing clostridia (8).

One ml quantities of 1 : 10 dilutions of the products were cultured in 0.05% sodium sulphite/iron citrate/agar (15) contained in oval cross section ('Miller-Prickett') tubes. The numbers of black colonies were determined after 1 to 3 days incubation at 32° C.

An adequate proportion of black colonies was pasteurized for one minute at 80° C. and then subcultured in poured plates of sulphite agar, contained in Petri-dishes made anaerobic by the use of pyrogallol/K_2CO_3/diatomaceous earth (15) as an oxygen adsorbent. The cultures obtained were tested for catalase reaction and aerobic growth. Positive cultures were discarded, while obligate anaerobes, if required, were further tested for gelatin liquefaction, nitrate reduction and fermentation of glucose, lactose, milk and mannitol under anaerobiosis (1).

(5) Aerobic sporebearing species (*B. cereus* etc.).

A 1 : 10 suspension of the food in 0.1% peptone/saline was heated for one minute at 80° C. and plated in duplicate, in an agar medium found to be more productive than ordinary tryptone/dextrose/yeast extract/agar, *viz.*, 1.5% tryptone/0.3% meat extract/0.5% dehydrated yeast extract/1.5% dehydrated peptonized milk/0.1% dextrose/agar, pH = 7.0; this medium will henceforth be called TDYM/agar. The numbers of colonies were counted after 1—3 days incubation at 32° C.

(6) Staphylococci (10).

Quantities of 0.05 ml of 1 : 10 dilutions of the products under investigation were streaked onto Chapman's 7.5% NaCl/agar. The numbers of yellow and white colonies were counted after 3 days incubation at 37° C.

An adequate proportion of cultures was tested for morphology and, if required, also for haemolysis, growth on tellurite/LiCl/glycine/agar (20) and coagulase.

(7) Total aerobic count.

One ml quantities of 1 : 10 dilutions of the products were plated in duplicate in TDYM/agar and colonies were counted after 1—3 days incubation at 32° C.

RESULTS AND DISCUSSION.

The results obtained have been collected in Table 1. It is clear, that not all samples met the tentative requirements, set out at the beginning.

The overall percentage of samples which complied with these standards varied rather widely, however, with the various types of precooked commodities: 100% for corn flakes and rice, 96% for baby cereals, 94% for rolled oats, 91% for instant puddings, 89% for whole wheat, whereas 63% only of dehydrated, precooked mashed potatoes and vegetables conformed to the suggested standard.

The deficiencies of the samples were not very serious though, as Salmonellae were not detected in any of the 85 samples. Regarding the other criteria, an average between 91 and 97% of all samples met the requirements for *Enterobacteriaceae*, faecal streptococci, sulphite reducing clostridia, *Staph. aureus* and aerobic colony count. In fact, deficiencies concerned mainly the numbers of aerobic spores.

Although there seems to be no reason why the bacteriological quality of dehydrated precooked potatoes and vegetables should be worse than that of instant cereals, nevertheless these foods can be considered separately, because in contrast to precooked cereals, they will generally be heated to a temperature between 70° and 85 °C. in the course of reconstitution. If these products were left out of the statistics, and if at the same time aerobic spores

TABLE 1.

Results of complete bacteriological analysis of 85 samples of dehydrated precooked ('instant') foods.

Commodity	Number of samples tested	Percentage of samples, containing the specified numbers per gramme of viable bacteria of the indicated groups														
		Salmonella per ca 25 g	*Enterobacteriaceae*			Faecal streptococci		Sulphite-reducing clostridia		*Staph. aureus*		Aerobic sporebearing cells				Total aerobic count	
			<10	10 10^2	$>10^2$	$<10^2$	10^2–10^3	$<10^2$	10^2–10^3	$<10^2$	10^2–10^3	$<10^2$		10^2 10^3	10^3–10^4	$<10^4$	10^4–10^5
Baby cereal	29	0	90	10	0	97	3	100	0	100	0	83		17	0	100	0
Cocoa	1	0	100	0	0	100	0	100	0	100	0	100		0	0	100	0
Corn flakes	5	0	100	0	0	100	0	100	0	100	0	100		0	0	100	0
Potatoes + vegetables	10	0	90	10	0	30	70	90	10	60	20 [1]	0		10	60 [2]	70	30
Pudding	10	0	100	0	0	100	0	100	0	80	20	60		40	0	100	0
Rice, whole	5	0	100	0	0	100	0	100	0	100	0	100		0	0	100	0
Rolled oats	10	0	90	10	0	100	0	90	10	100	0	80		20	0	100	0
Wheat, whole	15	0	80	13	7	100	0	93	7	100	0	47		47	6	100	0
Total and average	85	0	94	5	1	91	9	97	3	93	5	71	88	17	8	96	4
Same, excluding potatoes and vegetables			94	—	—	>99	—	98	—	97	—	81	99	18	—	100	—

[1] 20% of samples between 1 and 2 × 10^3. [2] 30% of samples between 1 and 2 × 10^4.

were allowed up to 10^3 per gramme, $\geqslant 94\%$ of all instant samples tested complied with the requirements.

CONCLUSIONS AND RECOMMENDATIONS.

From this study it appears, that in general manufacturers of instant cereals and puddings can now meet the following microbiological requirements: absence of Salmonellae in about 25 grammes, *Enterobacteriaceae* <10/gramme, faecal streptococci, sulphite-reducing clostridia and *Staph. aureus* $<10^2$/gramme, aerobic spores $<10^3$/gramme and total aerobic count $<10^4$/gramme.

If a little more care is taken in the preparation and handling of mixtures of dehydrated precooked vegetables and mashed potatoes, these commodities could also meet the suggested standards.

It may be useful to consider, both in the light of the literature (19, 17) and with reference to the results obtained in this study, which method of bacteriological examination should be recommended for the practical control of instant foods. It seems reasonable to limit the tests for the present, to the detection of Salmonellae and those which are normally carried out in the bacteriological examination of liquid milk or milk powder for example, *viz.*, enumeration of coliform bacteria, or rather (13) *Enterobacteriaceae*, and a total colony count. In order to check post-processing contamination it might be considered necessary to include a count of aerobic sporebearing cells, because if the total colony count considerably exceeded the spore count, this might indicate, with these dry products, that contamination had occurred after thermal processing. In the material examined by us 80% of all samples and 83% of samples excluding the dehydrated, precooked potatoes and vegetables satisfied the requirement that the total count was not significantly higher than the spore count.

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(From the Laboratory of Hygiene, University of Amsterdam).

A NEW METHOD OF THE REPORTING OF PHAGE PATTERNS OF *STAPHYLOCOCCUS AUREUS*

by

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(Received March 19, 1959).

Phage typing of Staphylococci is a complicated matter because many *Staphylococcus aureus* strains are sensitive not to one but to a number of phages. Most strains can be identified by the phage pattern they show when tested with the series of internationally recognised standard phages. These are used in routine test dilution (R.T.D.). This is the minimal phage concentration which gives complete lysis with the propagating strain (see Williams and Rippon, 1952).

Sometimes, however, it remains difficult to decide whether strains are identical or different. To some extent this is caused by the fact that only part of the information we can glean from the plate can be recorded in the patterns as they are now internationally in use. Moreover, it is sometimes difficult to compare patterns obtained by different laboratories. The reason for this is that only those phages which in R.T.D. strength give complete lysis or incomplete but still strong lysis, i.e. more than 50 plaques, are recorded. So the reported formula may be different if in another laboratory, due to biological and technical variation, some phages give just less than strong lysis with the same strain, or another phage reacts with just more than 50 plaques. Besides this, the relative strength of the various reactions is not taken into account. In order to overcome these difficulties as far as is possible, we suggest another way of reporting.

In our laboratory since 1952 a more extensive method of report-

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[1] Working with a grant from the Gezondheidsorganisatie T.N.O.

ing phage reactions has been used. This has proved to be very valuable for epidemiological studies.

The fundamental idea is that all visible phage reactions are recorded and reported in order of conspicuity. The recorded phage numbers are separated by commas. Besides, we use two oblique strokes, the first indicating the point between those phages which give complete lysis and those which do not. The second is put between those which are still easily visible and those reactions which are seen only at a second glance. The last point corresponds broadly to the conspicuity of 20 plaques of small size; in case of large plaques we decided to put reactions of less than about 15 plaques behind the second stroke. A colon may be put between the last of the ++ reactions and the first of the + reactions to separate the strong (more than 50 plaques) and weaker reactions in agreement with international use. Strain N.C.T.C. 8319 (propagating strain phage 3A) which generally is designated as 3A+, shows with our way of recording the following pattern 3A sp./ : 55,71 / 3B, 3C.

These weak reactions, even those consisting of one real plaque, truly belong to the phage pattern of a given strain. This can easily be proved by testing the same strain with phages in 10 or 100 R.T.D. strength. Moreover, they are reasonably consistent, sometimes surprisingly so, as may be seen from duplicate tests of one culture, from tests on successive cultures from one source and from retesting a culture after some months. Of course, the weak reactions behind the second stroke do not always turn up in the R.T.D. More concentrated phages, however, may then show the original pattern.

The differences between the results of various tests of the same strain are usually easily recognised to be chance occurrences. The differences between duplicate typings are sometimes a reversal in order of two or three phages, or a weaker reaction all over, with the phages in the same order of conspicuity. Sometimes several of the reactions in the "tail" of the formula are missing at R.T.D. strength but this is not confusing; if confirmation is necessary these become visible with 10 or 100 R.T.D.

If more phages give complete lysis the order of strength is unknown. Retyping this strain the next day, using the same broth culture (Hood, 1953) with 1/100 R.T.D. of these phages only, enables us to put these in order of strength too.

In cases where this is important for epidemiological purposes one can identify a strain more exactly by this new method than is possible with the one which is now in general use.

Fig. 1 gives part of the results of the typing of strain N.C.T.C. 8352 (propagating strain phage 70) with the international set of typing phages in R.T.D. With our notation the following formula is obtained 70/ : /73, 47, 53, 81, 42E, 7, 6, 54, 75. The phages behind the second stroke all give strong lysis when used in 1000 R.T.D., while no other phages are reacting in this way.

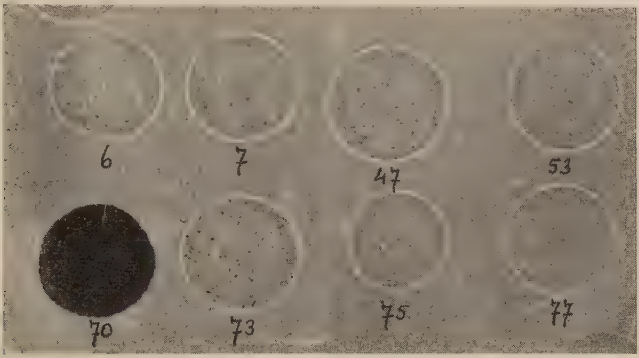

Fig. 1. Strain N.C.T.C. 8352 (P.S. 70) typed with phages in R.T.D. (magnification 1½×). The very small plaques of phage 75 cannot be seen on the reproduction. On the plate they are discernable with a hand lens or with the naked eye with reflected light.

We attach so much importance to the so-called tail of the phage pattern because this enables us to mark special strains, which in epidemiological studies may be of paramount importance. The following formulas give an example of strains we met in our longitudinal surveys of healthy people and which showed constant differences.

Strains which in the official connotation would have been judged to be identical - showing the pattern 29/52/80+ - were isolated repeatedly from a physician and a nurse in a maternity hospital. The minor phage reactions, however, showed a constant difference between the strains of these two people. Twelve times a strain was isolated from the physician during a period of 12 months which we noted as 29 / 52, 80 : 52A, 79, 77 / 75, 70, 53, 54. From the nurse in a period of 5 months a strain was cultured seven times with the phage pattern 29 / 52, 80 : 52A, 79 / —. The official connotation would have led us erroneously to the supposition that both were carrying the same strain. During the same period a strain with the phage pattern 29 / 52, 80 : 52A, 79 / 81, 73, 42E was present among the staff. This strain also could only be differentiated by its „tail".

If all strains as routine should be tested in addition with 100 or 1000 R.T.D. approximately the same information should be obtained. However, our method takes less time and less material.

Even with our method of recording not all available information appears in the report. For example, the size of the plaques is not mentioned. For identifying a strain, however, the new method gives additional information which is sometimes useful for the epidemiologist.

The value of the various minor reactions can be judged only by the expert in the same way as an X-ray picture gives all available information only to the insider.

Summary.

A new method of recording the phage pattern of *Staphylococcus aureus* is suggested. The phage numbers are given in order of strength of the various reactions. The stability of minor reactions gives valuable information for epidemiological purposes.

Acknowledgements.

We wish to express our thanks to Dr R. E. O. WILLIAMS for providing us with the phages and their propagating strains and for giving much additional information. To Miss J. WILLEMS, Miss N. E. EDEL and Miss G. R. VAN RHIJN we are very grateful for their technical help.

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OXIDATIVE POSSIBILITIES IN THE CATALASE POSITIVE *ACETOBACTER* SPECIES

by

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(Received May 16, 1959).

INTRODUCTION.

The genus *Acetobacter* was divided by FRATEUR (11) into four groups: the peroxydans, oxydans, mesoxydans and suboxydans group. This nomenclature originated from the earlier work of KLUYVER and DE LEEUW (20) and VISSER 'T HOOFT (26) who had compared the oxidations of various *Acetobacter* strains and was narrowly related to KLUYVER's general concept of the fundamental unity of life.

The division of the genus *Acetobacter* was based on a number of characters which at that time were interpreted as an expression of different "oxidizing power" which was thought to be low in suboxydans and high in peroxydans. Thus the suboxydans group was characterized by oxidations which led to the first oxidation products (e.g. ketoses from polyalcohols, ketogluconic acid from glucose) whereas the more oxidizing species did not form these intermediates but oxidized the substrates to completion. This view is still accepted in some modern textbooks of bacteriology (25).

However, it became clear from the work of TANENBAUM (24), ATKINSON (3) and DE LEY (5), that *A. peroxydans* is unable to attack glucose. Furthermore HAUGE, KING and CHELDELIN (13) and CUMMINS, KING and CHELDELIN (4) showed that *A. suboxydans* can oxidize some ketoses and polyalcohols to completion. Both facts are in contrast with the accepted view about the differences in "oxidizing power" within the genus *Acetobacter*.

The concept of oxidizing power is difficult to fit into current views about bacterial metabolism. In retrospection the term stands obviously for at least two phenomena:

a. the capability to oxidize a given substrate to completion;
b. the number of substrates that can be oxidized.
The facts should therefore be reconsidered in terms of the oxidative pathways involved (Krebs cycle, pentose phosphate cycle) and of the number of substrates that can be fed into these cycles (fig. 3, 5, 8).

In the present paper the oxidation of a number of substrates by resting cells of 20 different *Acetobacter* strains are reported. The results show that — except in the suboxydans group — all strains oxidize acetate, the C_4 dicarboxylic acids and α-ketoglutarate. No citric acid cycle is present in strains of the suboxydans group and in this sense suboxydans may be said to have a low "oxidizing power". However the number of substrates that can be fed into the pentose cycle increases from peroxydans to suboxydans. In this sense the suboxydans group has the highest oxidizing power. We feel however that the concept of "oxidizing power" should be dropped completely.

DESCRIPTION OF STRAINS.

Table 1 shows the origin of the strains used. New isolates were identified, according to FRATEUR (11).

A. ascendens N.C.I.B. 4937 and N.C.I.B. 8163 slowly produced acid on yeast extract-glucose-$CaCO_3$ slants and did not grow on Hoyer's medium. These strains really seem to belong in the rancens group.

Gluconacetobacter liquefaciens was found to be an addition to FRATEUR's ten basic species of *Acetobacter*. The "oxydogram" of this organism showed, that it could convert glycerol to dihydroxyacetone, mannitol to fructose and sorbitol to sorbose. On yeast extract-Ca-lactate strong irisation occurred and on yeast extract-ethanol-$CaCO_3$ acid was produced at first but on prolonged incubation $CaCO_3$ was formed again. Consequently this strain belongs to the mesoxydans group. The strain did not produce cellulose. In Hoyer's medium growth was slow; it was however transferable. The slow growth on this medium makes a classification as *A. aceti* or as *A. mesoxydans* doubtful. However on yeast extract-glucose-$CaCO_3$ a deep brown colouration was produced, similar to the one produced by *A. melanogenum* in the suboxydans group. Because of this property *Gluconacetobacter liquefaciens* might be considered as a new species in the mesoxydans group.

TABLE 1.

Strain	Origin
1. Oxydans group.	
A. ascendens (a)	Techn. Univ. Delft.
A. lovaniense (a)	,, ,, ,,
A. rancens (a)	,, ,, ,,
A. rancens (isol. 1)	Isolated from beer.
A. rancens variety (b)	Nat. Coll. Ind. Bacteria.
N.C.I.B. 4937 and N.C.I.B. 8163	*A. ascendens* 4937 and 8163 (see text).
A. rancens (isol. 29)	Isolated from beer.
A. rancens var. *turbidans* (isol. 26)	,, ,, ,,
A. rancens var. *saccharovorans* (a)	Techn. Univ. Delft.
A. rancens (isol. 10)	Isolated from beer.
A. rancens var. *turbidans* (isol. 16)	,, ,, ,,
A. rancens (isol. 24)	,, ,, ,,
2. Suboxydans group.	
A. suboxydans (isol. 18)	Isolated from beer.
A. suboxydans var. *biourgianum* (isol. 22)	,, ,, ,,
A. suboxydans var. *biourgianum* (isol.2)	,, ,, ,,
A. melanogenum (a)	Techn. Univ. Delft.
3. Mesoxydans group.	
A. aceti (a)	Techn. Univ. Delft.
A. mesoxydans (a)	,, ,, ,,
A. xylinum (b)	Univ. of California, Davis.
A. mesoxydans var. *saccharovorans* (a)	Techn. Univ. Delft.
Gluconacetobacter liquefaciens (b) (Nov. spec. in the mesoxydans group; see text).	Univ. of Tokyo.

The strains marked with (a) were received by the courtesy of Prof. T. O. WIKÉN; the strains marked with (b) by the courtesy of Dr J. DE LEY. The Delft strains are the original strains described by FRATEUR (11).

Of the isolated strains the numbers 10, 16, 18 and 24 produced acid on yeast extract-glucose-$CaCO_3$, afterwards $CaCO_3$ was formed again, shown by irisation. This indicates, that the (gluconic) acids formed are completely oxidized.

A more detailed description of the physiological properties of these strains will be published later (23).

EXPERIMENTAL.

Cell suspensions were generally prepared from cultures on a solid medium containing 10% glucose, 1% yeast extract, 2½% agar and 3% $CaCO_3$ (in suspension) in Roux flasks. *A. xylinum* (Davis) and *A. rancens* variety N.C.I.B. 4937 were grown, however, on a medium containing 2% ethanol, 1% yeast extract, 2½% agar and 2% $CaCO_3$, to avoid difficulties in preparing suspensions from glucose grown cells of *A. xylinum* and because of the poor growth of N.C.I.B. 4937 on the glucose containing medium. *A. ascendens* was cultured on a medium, composed of a wort agar, previously fermented by baker's yeast. This strain showed weak growth on the $CaCO_3$ containing medium, since it had a lower pH optimum.

Roux flasks, containing 150 ml of one of these three media, were inoculated and incubated at 30° C. for 48 hours. The cells were then washed from the agar surface with M/100 phosphate buffer pH 6.0, collected by centrifugation and washed twice with the same buffer. The cell yield per Roux flask varied for the different strains from 0.3—1.4 g wet weight. After the second washing the cells were suspended in M/15 phosphate buffer pH 6.0 to a concentration of about 50 mg (wet weight) per ml.

Per Warburg vessel 1.7 ml suspension was used; 0.2 ml substrate (10 μ moles) were tipped in from the side bulb. The evolution of CO_2 was measured by the direct method and corrected for CO_2 retention in the buffer. Ketogluconates were detected by descending paper chromatography as described previously (7). The o-phenylene-diamine spray (21) was used to detect ketogluconates. Good separations of 2- and 5-ketogluconate and 2.5-diketogluconate could be achieved in this way. After heating for 3 min. at 95—100°C. 2.5-diketogluconate is giving a yellow-brown spot, with yellow fluorescence. Gluconate was estimated by the method of HESTRIN (14). 2-Ketogluconate by the method of LANNING and COHEN (21). Cell free extracts were prepared by grinding with alumina. Enzyme determinations were performed as described previously (7).

RESULTS.

The oxygen consumption of all strains on various substrates is given in tabular form together with some of the FRATEUR characters. The substrates are arranged so as to appear under the FRATEUR character with which they seem related. Oxygen consumption is given as moles O_2 per mole substrate, corrected for endogenous respiration. In many cases with slow oxygen consumption the reaction was not finished at the end of the experiment (3—4 h) which is indicated by the prefix >. Irisation on lactate indicates the oxidation of lactate to CO_2 and can be considered as presumptive evidence of the presence of a citric acid cycle.

Irisation on ethanol indicates the possibility to activate acetate, and thus to feed acetate into the citric acid cycle. A positive oxydogram on glycerol indicates dihydroxyacetone production and is often (erroneously) thought to imply the oxidation of all polyalcohols.

Irisation on glucose proves the formation of CO_2 from gluconate; it will be argued, that it is an indication of the presence of gluconokinase, by which gluconate can be fed into the pentose cycle.

A. Oxydans group.

The results obtained with the strains of the oxydans group are shown in table 2.

1. Citric acid cycle.

All strains show irisation on lactate and are able to metabolize the C_4 dicarboxylic acids and α-ketoglutarate. An example of the oxidation of these substrates by one of the strains of this group is shown in fig. 1. Citrate was never oxidized at pH 6.0; DL-isocitrate very slowly. At pH 3.5 citrate was actively oxidized by most strains. In cell free extracts of *A. rancens* (isol. 10) a DPN specific isocitrate-dehydrogenase was demonstrated. With citrate as substrate these extracts also reduced DPN.

Considering these results and in agreement with the findings of KING, KAWASAKI and CHELDELIN (18) with *A. pasteurianum* (a rancens variety) the presence of this cycle in all strains of the oxydans group can be assumed.

All strains show irisation on ethanol-$CaCO_3$ and on Ca-lactate. Both these substrates were oxidized rapidly. In some strains a sharp inflection in the respiration curve for ethanol occurred after the uptake of 1 mole O_2 (fig. 2). Such an inflection may be due either to a weak condensing enzyme or to a weak acetate activating system. As most of these strains oxidized DL-lactate and pyruvate to completion without inflections in the oxidation curves, the second possibility seems the more important one.

TABLE 2.

Oxidations by resting cells of some strains of the oxydans group. (explanation see text).

Strain	*A. ascendens* Delft	*A. lovaniense* Delft	*A. rancens* Delft	*A. rancens* (isol. 1)	*A. rancens* var. N.C.I.B.4937	*A. rancens* (isol. 29)	*A. rancens* var. *turbidans* (isol. 26)	*A. rancens* var. *saccharovorans*	*A. rancens* (isol. 10)	*A. rancens* var. *turbidans* (isol. 16)	*A rancens* (isol. 24)
Irisation on Ca-lactate and ethanol-CaCO$_3$	++	++	++	++	++	++	++	++	++	++	++
Ethanol	> 1.8	> 2.2	> 1.4	> 1.4	2.4	2.6	2.6	> 1.8	3.0	2.8	2.1
Acetate	N.T.	N.T.	> 0.4	> 0.4	1.8	N.T.	N.T.	N.T.	1.9	2.0	N.T.
DL-lactate	N.T.	2.6	2.5	> 2.2	> 1.7	2.8	2.1	> 2.1	2.4	2.6	2.1
Pyruvate	> 0.9	> 1.6	> 1.6	> 1.4	> 0.9	N.T.	1.7	> 1.2	1.8	1.9	N.T.
Succinate	N.T.	2.3	> 2.1	N.T.	> 2.6	N.T.	3.1	> 0.6	> 2.6	> 2.7	N.T.
Fumarate	> 1.0	> 1.0	> 1.6	N.T.	> 2.1	2.7	> 1.1	> 0.7	> 1.1	> 1.8	> 2.1
DL-malate	N.T.	N.T.	> 0.8	N.T.	> 1.4	N.T.	N.T.	> 0.5	> 0.8	> 0.6	N.T.
α-ketoglutarate	N.T.	N.T.	> 0.8	N.T.	> 2.1	N.T.	> 1.8	> 0.1	> 0.2	> 0.7	N.T.
Oxydogram on glycerol	—	—	—	—	—	—	—	—	—	—	—
Glycerol	> 0.4	> 1.3	> 1.9	0	> 0.1	2.6	2.1	> 1.6	2.4	3.0	1.9
Mannitol	0	0	0	0	0	0	> 0.5	0	> 0.1	> 0.1	> 0.1
Sorbitol	0	0	> 0.1	N.T.	0	0	> 0.1	> 0.2	> 0.1	0	0
Erythritol	0	0	0	N.T.	0	0	0	> 0.1	0	0	0
Dioxyacetone	> 0.2	> 0.6	> 0.4	> 0.1	> 0.1	N.T.	1.1	> 0.8	> 1.1	2.0	N.T.
Fructose	0	0	> 0.1	> 1.0	0	0	> 0.1	0	> 0.1	> 0.1	> 0.1
L-sorbose	N.T.	0	0	N.T.	N.T.	N.T.	0	N.T.	0	0	N.T.
Acid on glucose-CaCO$_3$	—	+	+	+	+	+	+	+	+	+	+
Irisation on glucose-CaCO$_3$	—	—	—	—	—	—	—	+	+	+	+
Glucose	0	0.5	0.5	0.5	0.4	1.1	1.4	> 1.5	> 3.2	> 2.9	2.6
Gluconate	0	0	0	> 0.1	0	0.5	0.5	> 1.0	> 2.6	> 2.2	2.1
2-ketogluconate	0	0	0	0	0	0	> 0.1	> 0.1	0.2	0.4	0
5-ketogluconate	0	0	0	0	0	0	0	0	0	0.2	0
2,5-diketoglonate	N.T.	N.T.	N.T.	N.T.	N.T.	N.T.	N.T	N.T.	0	0	N.T.
5-ketogluconate + H-donor	N.T.	N.T.	0	N.T.	N.T.	N.T.	0	N.T.	0	0.2	N.T.

N.T. = not tested.

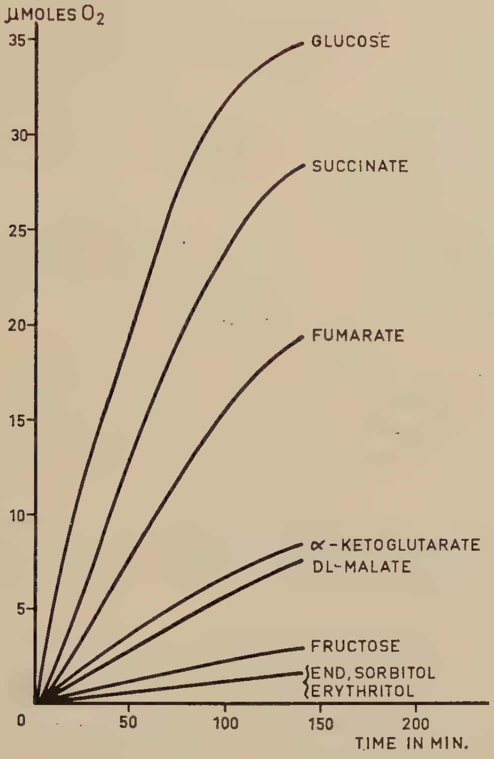

Fig. 1. Oxygen consumption by resting cells of *A. rancens* var. *turbidans* (isol. 16) with various substrates. Glucose and citric acid cycle intermediates are readily oxidized, while fructose, sorbitol, erythritol (and mannitol) are not.

2. The oxidation of polyalcohols and ketoses.

In agreement with the definition of the group none of the strains show a positive oxydogram on glycerol. *A rancens* (isol. 1 and N.C.I.B. 4937) indeed do not oxidize glycerol or dihydroxyacetone (DHA). The other strains metabolize these substrates actively and in most cases the O_2 uptake with glycerol was proceeding far beyond the dihydroxyacetone stage. It might be argued that these strains do form dihydroxyacetone from glycerol but that the oxydogram is negative due to the rapid breakdown of the DHA formed. However in cell free extracts of *A. rancens* (isol. 10) no enzyme for the conversion of glycerol to DHA could be detected but the presence of an active glycerokinase and DHA-kinase was observed. For this strain DHA is not an intermediate in glycerol breakdown.

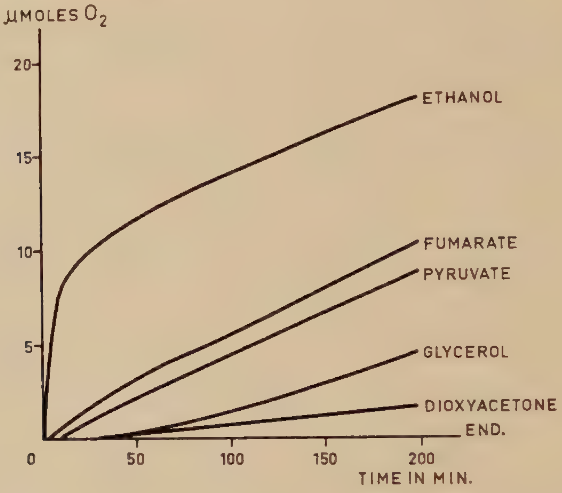

Fig. 2. Oxygen consumption by resting cells of *A. ascendens* (Delft). Note the sharp inflection in the respiration curve for ethanol. Glucose, gluconate, ketogluconates, fructose and the polyalcohols (except glycerol) were not oxidized.

Thus glycerol is metabolised only after an initial phosphorylation. Considering the observation that even suboxydans strains — for which DHA is an intermediate in glycerol breakdown and who do oxidize DHA — always accumulate large quantities of DHA it can be concluded that none of the oxydans strains is capable of oxidizing glycerol to DHA.

The other polyalcohols and ketoses were only oxidized in some cases. *A. rancens* var. *turbidans* (isol. 26) was readily oxidizing mannitol. The oxydogram of this strain on mannitol is positive. Thus this strain is capable to convert mannitol in fructose. *A. rancens* var. *saccharovorans* slowly oxidized sorbitol and the oxydogram on sorbitol showed that traces of a reductive substance had been formed. In all other cases the polyalcohols were not or very slowly oxidized. Fructose was only oxidized by *A. rancens* (isol. 1). The O_2 uptakes with the other strains were very low. L-sorbose was not oxidized by the oxydans strains.

It can be concluded that the oxidation of polyalcohols and ketoses is not a general property of the oxydans group, though occasionally slow oxidation is observed with some of these substrates.

3. Glucose metabolism.

No glycolytic system seems to be present in the strains of this group as none of the strains produced CO_2 from glucose under anaerobic condition, whereas CO_2 was readily produced from pyruvate. As far as glucose oxidation is concerned the strains fall — with two exceptions — into two major groups:

a. strains which oxidize glucose to gluconic acid with an uptake of 0.5 mole O_2 but are unable to break down gluconate;

b. strains which give irisation on glucose, oxidize glucose with a high O_2 uptake and also show an appreciable O_2 uptake with gluconate.

A. ascendens (Delft) was unable to oxidize glucose at all. As this strain gave optimal growth at lower pH glucose oxidation was also studied at pH 3.5 (citrate-phosphate) but no oxygen uptake was observed.

Ad. a. *A. lovaniense, A. rancens* (Delft and isol. 1) and *A. rancens* variety N.C.I.B. 4937 oxidized glucose with the uptake of 0.5 moles O_2/mole. No CO_2 was produced. Glucose was converted quantitatively to gluconate. None of these strains metabolized gluconate or the ketogluconates, thus 2-ketogluconate was not produced from gluconate. The strains of this group are unable to feed glucose into the pentose cycle.

The strains *A. peroxydans* (Davis) and *A. peroxydans* N.C.I.B. 8138 (most probably both strains are indeed rancens varieties) studied by De Ley (5) also belong to this group as far as glucose metabolism is concerned.

A. rancens (isol. 29) and *A. rancens* var. *turbidans* (isol. 26) take an intermediate position. The first strain oxidizes glucose with an uptake of 1.1 mole O_2, the second with 1.5 mole O_2. Both strains oxidize gluconate with about 0.5 mole O_2. They are forming 2-ketogluconate as was shown by paperchromatographic and colorimetric analysis of the supernatants of the glucose- and gluconate oxidation. 2-Ketogluconate was not metabolized. The oxidation curves for *A. rancens* var. *turbidans* (isol. 26) indicate however that not all glucose is metabolized over gluconate. Part of the glucose is probably phosphorylated and fed into the pentose cycle as occurs in strains of the mesoxydans and suboxydans group. Thus the oxidation curves for glucose and gluconate by this strain are about the same as those shown in fig. 7 for *A. aceti* (Delft).

Ad. b. The 4 strains, showing irisation on yeast extract glucose-

$CaCO_3$ slants give much higher O_2 uptakes with glucose and gluconate than the preceding strains. *A. rancens* var. *saccharovorans* gives a respiration curve for glucose with a sharp inflection after the uptake of 0.5 mole O_2 indicating that all the glucose was metabolized via gluconate. With the three other strains no such inflections were observed (fig. 1). *A. rancens* var. *saccharovorans* and *A. rancens* (isol. 29) are unable to oxidize ketogluconates. The two other strains, *A. rancens* var. *turbidans* (isol. 16) and *A. rancens* (isol. 10) gave relatively low and varying values for the O_2 uptake with ketogluconates. As Fewster (10) reported that *A. suboxydans* oxidizes 5-ketogluconate only in the presence of another oxidizable substrate the influence of other substrates on ketogluconate oxidation was studied. In none of the tested strains of the oxydans group, the O_2 uptake with ketogluconates was increased by the addition of DHA, pyruvate or ethanol. After the O_2 uptake on the ketogluconates had ceased most of the ketogluconate was still present in the supernatant as could be shown by paperchromatography. We suggest that the low O_2 uptake is due to impurities and assume that this group of strains cannot attack ketogluconates.

In cell free extracts of all strains which gave irisation on glucose-$CaCO_3$ gluconokinase could be detected whereas this enzyme was absent in extracts from all strains not showing irisation. It is assumed that by means of the gluconokinase these strains are able to feed gluconic acid into the pentose cycle.

The pentose cycle seems to be present in most *Acetobacter* strains. The presence of the cycle in the mesoxydans and suboxydans groups will be argued below. De Ley (5) showed its presence in *A. peroxydans*, though in this species the cycle may be only functional in the synthesis of cell components, as sugars cannot be fed into the cycle.

In the oxydans group the pentose cycle has to be present in the strains that show irisation on glucose. The high oxygen uptake with glucose and gluconic acid seems to form presumptive evidence in this respect, the more so as no glycolytic system could be detected. In cell free extracts of *A. rancens* (Delft and isol. 10) the G-6-P- and 6-PG-dehydrogenases were shown to be present.

Our data permit a preliminary picture of the possibilities of carbohydrate metabolism in the oxydans group which is tentatively expressed in the diagram of fig. 3.

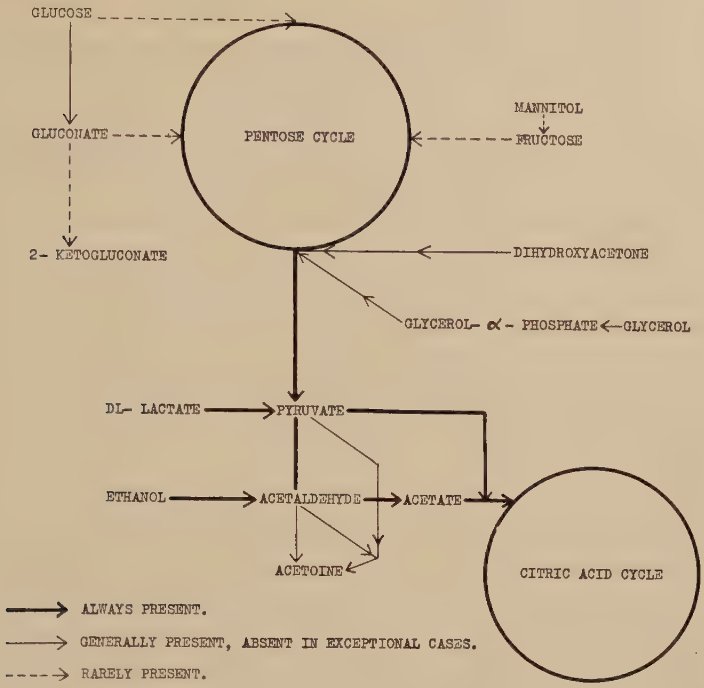

Fig. 3.
Carbohydrate metabolism of *Acetobacter* strains of the oxydans group.

B. Suboxydans group.

The results of the strains of the suboxydans group are shown in table 3. In this table are included the results of FEWSTER (10) with glucose grown cells of *A. suboxydans* A.T.C.C. 621 and of HAUGE, KING and CHELDELIN (13) and CUMMINS, KING and CHELDELIN (4) with glycerol or sorbitol grown cells of the same strain. Some of the results with *A. suboxydans* (isol. 18) were reported already by DE LEY and STOUTHAMER (7).

1. Citric acid cycle.

Ethanol, pyruvate and DL-lactate are only oxidized to acetate. Acetate is not metabolized. The C_4 dicarboxylic acids, α-ketoglutarate, citrate and isocitrate are generally not oxidized.

The sole exception is *A. suboxydans* var. *biourgianum* (isol. 2) which oxidizes the C_4 dicarboxylic acids slowly and after a long induction phase. The citric acid cycle seems to be absent in all

TABLE 3.

Oxidations by resting cells of some strains of the suboxydans group. (explanation see text)

Strain	*A. suboxydans* (isol. 18)	*A. suboxydans* ATCC 621	*A. suboxydans* var. *biourgianum* (isol. 22)	*A. suboxydans* var. *biourgianum* (isol. 2)	*A. melanogenum* (Delft)
Irisation on Ca-lactate	—	—	—	—	—
and ethanol-$CaCO_3$	—	—	—	—	—
Ethanol	0.9	1.0	0.8	1.0	0.8
Acetate	0	0	0	0	0
DL-lactate	> 0.8	1.0	0.8	1.0	0.9
Pyruvate	> 0.4	0.5	0.4	0.4	0.4
Succinate	0	0	0	> 0.3	0
Fumarate	0	0	0	> 0.4	0
DL-malate	0	0	0	> 0.4	0
α-ketoglutarate	0	0	N.T.	0	0
Oxydogram on glycerol	+	+	+	+	+
Glycerol	0.8	2.2	0.5	1.8	0.7
Mannitol	4.3	4.3	4.3	> 0.1	4.6
Sorbitol	> 3.0	> 2.2	> 0.9	> 0.7	> 2.4
Erythritol	> 2.6	N.T.	> 0.7	2.1	> 1.0
Ribitol	0.5	0.5	> 0.3	> 0.1	0.5
Dioxyacetone	0	1.7	> 0.3	> 0.1	0
Fructose	4.2	4.2	> 2.0	> 0.1	3.9
L-Sorbose	> 1.9	> 1.4	0	> 0.2	> 0.3
Irisation on glucose-$CaCO_3$	+	+	—	—	—
Formation of 2,5-diketogluconate	—	—	—	+	+
Glucose	4.2	3.75	0.9	1.8	1.3
Gluconate	3.6	3.2	> 0.3	1.0	0.6
2-ketogluconate	3.4	0.5	0	0.5	0.5
5-ketogluconate	3.1	0	0	0	0
2.5-diketo-gluconate	2.6	N.T.	N.T.	0	0
5-ketogluconate + H-donor	—	3.0	N.T.	0	0

The data of *A. suboxydans* A.T.C.C. 621 are cited from FEWSTER (10) and from HAUGE, KING and CHELDELIN (13) and CUMMINS, KING and CHEIDELIN (4).

N.T. = not tested.

strains of the suboxydans group. This conclusion is in agreement with the observations of King and Cheldelin who found no citric acid cycle in *A. suboxydans* A.T.C.C. 621 (17).

2. The oxidation of polyalcohols and ketoses.

Mannitol and fructose are oxidized with an O_2 uptake of about 4 moles/mole substrate by most strains. This high O_2 uptake indicates that the pentose cycle is the main pathway of carbohydrate breakdown in the suboxydans group in accordance with the results of Hauge, King and Cheldelin (13) with *A. suboxydans* A.T.C.C. 621. *A. suboxydans* var. *biourgianum* (isol. 2) though not oxidizing mannitol and fructose shows a high O_2 uptake with glycerol which indicates that a pentose cycle is probably also present in this strain. Besides its presence was shown in *A. melanogenum* M.A. 6.2 by Katznelson (16) and in *A. suboxydans* (isol. 18) by De Ley and Stouthamer (7). In cell free extracts of *A. melanogenum* (Delft) we demonstrated the presence of G-6-P- and 6-PG-dehydrogenases.

Sorbitol was oxidized by all strains with an O_2 uptake of more than 0.5 moles O_2. The respiration curves showed a sharp inflection and often went on with the same slope as the curve for the oxidation of L-sorbose (fig. 4). The inflection occurred at a much higher level than 0.5 mole O_2. This might be explained by the oxidation of sorbitol to a mixture of D-fructose and L-sorbose as described by Cummins, King and Cheldelin (4) and Arcus and Edson (2) for *A. suboxydans* A.T.C.C. 621.

A. suboxydans var. *biourgianum* (isol. 2) does not oxidize fructose and forms only traces of fructose after 24 hr. incubation with mannitol.

The respiration curve for erythritol rises steeply to an oxygen consumption of 0.5 mole O_2. After a sharp inflection oxygen consumption continues at a lower rate. Presumably erythritol is oxidized quickly to erythrulose, which is than oxidized slowly. The pathways, by which L-sorbose and L-erythrulose are metabolized, are unknown. Because of the high O_2 uptakes, it is probable, that these compounds are transformed into intermediates of the pentose cycle. Ribitol was quickly oxidized with O_2 uptakes of about 0.5 mole indicating, that D-ribulose is not oxidized.

All strains show a positive oxydogram on glycerol. Indeed all strains oxidize glycerol, with a mean O_2 uptake of 1.2 mole. *A. suboxydans* var. *biourgianum* (isol. 2) oxidizes glycerol after an

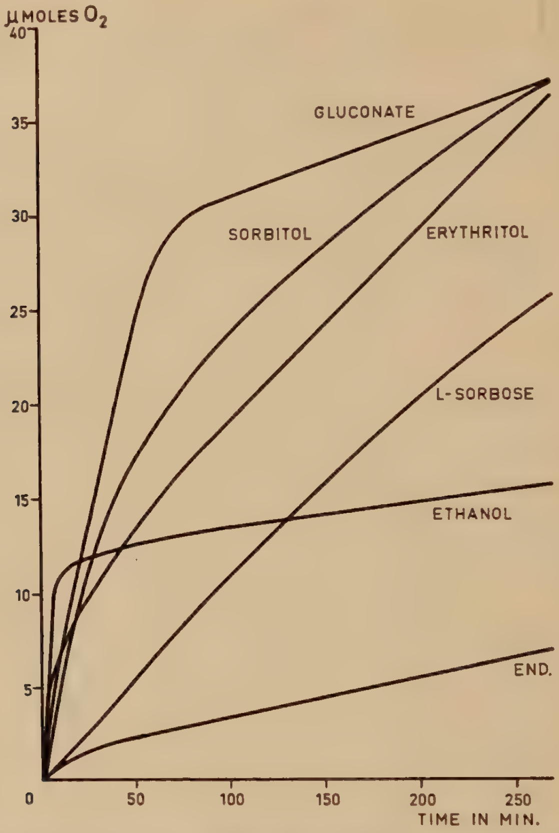

Fig. 4. Oxygen consumption by resting cells of *A. suboxydans* (isol. 18) with various substrates. Note the rapid oxidation of gluconate and polyalcohols and the incomplete oxidation of ethanol. The respiration curves for sorbitol and L-sorbose are obtaining the same slope, after much more than 0.5 mole O_2 has been consumed with sorbitol.

induction phase. *A. melanogenum, A. suboxydans* (isol. 18) and *A. suboxydans* var. *biourgianum* (isol. 2) do not oxidize DHA. The latter strain however was capable of oxidizing DHA in the presence of glycerol, with an uptake of 1.4 mole O_2/mole DHA. This explains the high O_2 uptake for glycerol by this strain and the absence of reducing substance in the supernatant after glycerol oxidation. Hauge, King and Cheldelin explained the induction phase in DHA oxidation observed with *A. suboxydans* A.T.C.C. 621 by an ATP requirement for starting the oxidation. The oxidative phosphorylation accompanying glycerol oxidation would facilitate

DHA phosphorylation and oxidation. The same explanation might be invoked for our results with *A. suboxydans* var. *biourgianum* (isol. 2).

3. Glucose metabolism.

In this group there is again a sharp distinction between strains which show irisation on glucose and strains which lack this property.

a. In the strains, which do not show irisation on glucose-$CaCO_3$, the O_2 uptakes for glucose and gluconate indicate, that glucose is partly converted into ketogluconates and partly metabolized by the pentose cycle, after phosphorylation by a hexokinase. This latter enzyme was shown to be present in cell free extracts of *A. suboxydans* (isol. 18) and *A. melanogenum*. FEWSTER (9) had already shown the presence of this enzyme in cell free extracts of *A. suboxydans* A.T.C.C. 621.

Paperchromatographic analysis of the supernatants of the oxidations of glucose, gluconate and 2-ketogluconate by *A. melanogenum* and *A. suboxydans* var. *biourgianum* (isol. 2), revealed that in both strains these substrates were oxidized to 2.5-diketogluconate. The main oxidation product of glucose and gluconate by *A. melanogenum* proved to be 5-ketogluconate however. KATZNELSON, TANENBAUM and TATUM (15) reported, that the characteristic brown coloration of *A. melanogenum* which occurs after a few days on glucose-$CaCO_3$ media is due to spontaneous decomposition of 2.5-diketogluconate. Because of this report *A. suboxydans* var. *biourgianum* (isol. 2) was incubated on yeast extract-glucose-$CaCO_3$ slants for long periods. After 3—5 weeks a brown coloration in the medium occurred. Still this strain was forming 2.5-diketogluconate as rapidly as *A. melanogenum*. This shows, that it is very improbable, that the brown coloration is due to spontaneous decomposition of 2.5-diketogluconate, because both strains are forming 2.5-diketogluconate at equal rates.

b. With the strains showing irisation on glucose-$CaCO_3$, the O_2 uptakes for glucose and gluconate, are much higher than in the former group.

In *A. suboxydans* (isol. 18) these compounds and 2- and 5-ketogluconate and 2.5-diketogluconate are oxidized with O_2 uptakes of 60 to 70% of the amount needed for complete oxidation. DE LEY and STOUTHAMER (7) showed, that the first step in the metabolism of 2- and 5-ketogluconate by this strain is a reduction to

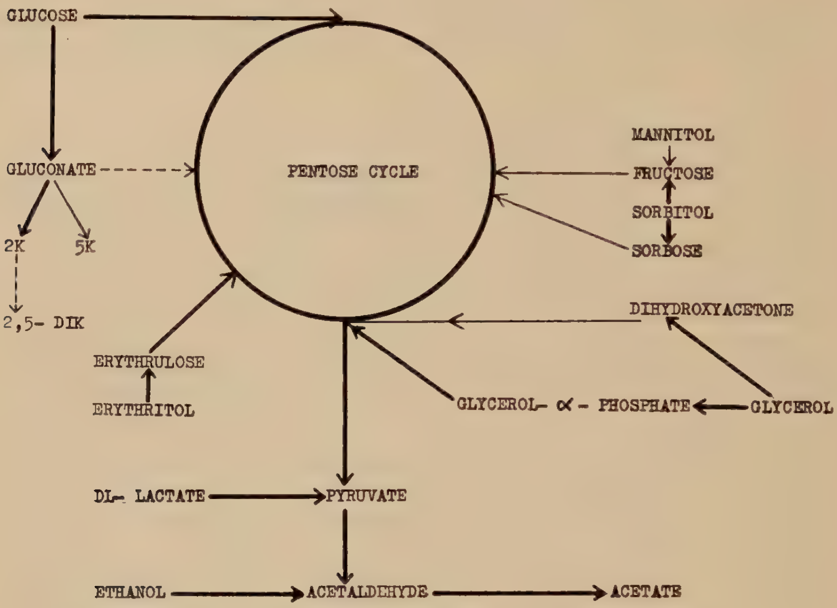

Fig. 5.
Carbohydrate metabolism of *Acetobacter* strains of the suboxydans group.

gluconate. 2.5-Diketogluconate was oxidized with an uptake of 2.6 moles O_2. We showed recently, that this compound is also metabolised after initial reduction to gluconate (23). In *A. suboxydans* (A.T.C.C. 621) 5-ketogluconate was only oxidized in the presence of an oxidizable substrate (10). This indicates that the 5-ketogluconate is again metabolized only after an initial reduction as in *A. suboxydans* (isol. 18). In *A. suboxydans* (A.T.C.C. 621) 2-ketogluconate is said to be oxidized to D-arabonate (10). However the evidence for the identification of this compound as D-arabonate was weak.

None of the strains of the suboxydans group produced CO_2 from glucose and fructose under anaerobic conditions, whereas with pyruvate 1 mole CO_2 was set free per mole substrate.

From these results the main features of carbohydrate metabolism in the suboxydans group can be derived, which are tentatively shown in fig. 5.

C. Mesoxydans group.

The results of the strains of the mesoxydans group are shown in table 4. In this table are included the results of De Ley and Schell with *A. aceti* Ch. 31 (cf. 8).

1. Citric acid cycle.

The metabolism of ethanol, lactate, pyruvate, acetate and citric acid cycle intermediates is the same as in the oxydans group. The enzymes of the citric acid cycle were demonstrated already in *A. aceti* by Rao (22). Resting cells of *A. mesoxydans* var. *saccharovorans* oxidized all citric acid cycle intermediates, except citrate. DL-isocitrate was oxidized with an uptake of > 0.4 mole O_2. In cell free extracts a DPN specific isocitric-dehydrogenase was detected. Citrate was also giving DPNH with this cell free extract. Thus the presence of a citric acid cycle can safely be assumed in this strain.

However, the last strain is not capable to oxidize acetate whereas DL-lactate and pyruvate were completely oxidized (fig. 6).

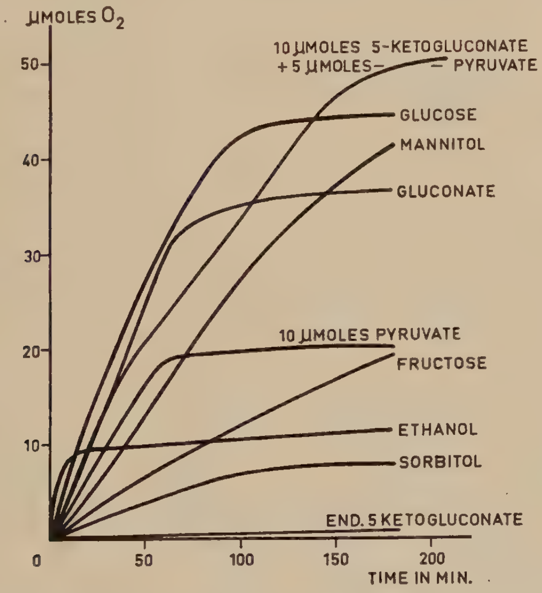

Fig. 6. Oxygen consumption by resting cells of *A. mesoxydans* var. *saccharovorans*, showing the complete oxidation of pyruvate and glucose; the incomplete oxidation of ethanol and the oxidation of 5-ketogluconate in the presence of pyruvate.

TABLE 4.

Oxidations by resting cells of some strains of the mesoxydans group. (explanation see text).

Strain	*A. aceti* (Delft)	*A. aceti* (Ch. 31)	*A. mesoxydans*	*A. xylinum* (Davis)	*A. mesoxydans* var. *saccharovorans*	*Gluconacetobacter liquefaciens*
Irisation on Ca-lactate	+	+	+	+	+	+
Irisation on ethanol-$CaCO_3$	+	+	+	+	—	+
Ethanol	2.4	2.4	>1.6	2.7	1.0	2.4
Acetate	2.1	>1.2	N.T.	N.T.	0	1.7
DL-lactate	2.2	2.4	>1.9	1.7	2.8	2.6
Pyruvate	1.8	2.0	>0.7	1.2	2.0	1.5
Succinate	>0.6	>1.2	>0.3	N.T.	>2.0	>1.5
Fumarate	>0.2	1.9	>0.6	N.T.	2.0	>1.4
DL-Malate	1.5	1.6 (L)	N.T.	N.T.	1.4	1.2
α-Ketoglutarate	>0.3	N.T.	>0.2	0	>1.1	>2.0
Oxydogram on glycerol	+	+	+	+	+	+
Glycerol	>2.2	>2.3	>1.2	>2.2	2.6	>1.9
Mannitol	>0.5	>1.5	>0.1	>1.3	5.1	>2.4
Sorbitol	>0.7	0	0	>0.4	0.8	>0.5
Erythritol	>0.6	>0.7	>0.2	>0.3	>0.9	>1.0
Ribitol	>0.2	N.T.	>0.1	N.T.	0.5	0.5
Dioxyacetone	>1.1	N.T.	>0.1	>0.7	1.8	>1.5
Fructose	>0.1	0	0	>0.4	>1.8	4.1
L-sorbose	0	0	0	N.T.	0	0
Irisation on glucose-$CaCO_3$	—	—	—	—	+	[2]
Glucose	>1.4	1.3	>0.9[1]	>1.6	4.5	>2.4
Gluconate	>0.3	0	>0.3[1]	>0.5	4.0	>2.0
2-Ketogluconate	>0.1	0	>0.1	>0.1	>2.6	>1.5
5-Ketogluconate	0	0	>0.1	0	0	3.4
2,5-diketogluconate	N.T.	N.T.	N.T.	N.T.	2.9	>1.0
5-ketogluconate + H-donor	0	—	—	—	3.8	—

The data with *A. aceti* Ch. 31 are cited from De Ley and Schell (8). N.T. = not tested.

[1] The values for the O_2 uptakes with glucose and gluconate by *A. mesoxydans* are the results obtained with old cells of this strain. For young cells higher values were found. With the other strains no differences in O_2-uptakes between young and old cells were noticed.

[2] Formation of brown coloration.

Probably the activating system for acetate is lacking. This result shows the limitations of the system for the classification of *Acetobacter* in Bergey's Manual of Determinative Bacteriology (7th Ed.). The main criterium in this classification system is the oxidation of acetate. With this system this strain would be classified as *A. suboxydans*! The criteria in the classification system of FRATEUR are the complete oxidation of lactate and of acetate. A strain, which is having one of these capabilities, should not be classified as *A. suboxydans.*

2. The oxidation of polyalcohols and ketoses.

Polyalcohols are readily oxidized by most strains, but not by all as in the suboxydans group. *A. mesoxydans* does not oxidize mannitol and sorbitol. *A. aceti* Ch. 31 does not oxidize sorbitol. For those strains which do oxidize polyalcohols the oxygen consumption is generally lower than for strains of the suboxydans group, besides the oxidations are much slower. L-sorbose is not oxidized by these strains. Fructose is oxidized by three strains, but not by *A. aceti* (Delft and Ch. 31) and *A. mexoxydans.*

In general the number of polyalcohols that can be fed into the pentose cycle is smaller than for the suboxydans group and the oxygen consumption is somewhat lower (see for instance fig. 7). Glycerol is oxidized — at least partly — via DHA as shown by the oxydogram. Dihydroxyacetone is oxidized by most strains.

3. Glucose metabolism.

Glycolysis is again absent as is shown by the absence of CO_2 evolution from glucose and fructose under anaerobic conditions and active decarboxylation of pyruvate in a N_2 atmosphere.

a. Strains which do not show irisation.

Oxidation of glucose by strains, which do not show irisation on yeast extract-glucose-$CaCO_3$ slants is comparable to the oxidation by suboxydans strains. Glucose is partly oxidized to ketogluconate (in *A. aceti* Ch. 31 only to gluconate) and partly fed into the pentose cycle by the hexokinase (fig. 7). For old cells (48 hours or more) of *A. mesoxydans* however the picture is more comparable with oxydans strains. Glucose is rapidly oxidized to gluconate which is then slowly converted to 2-ketogluconate, as shown by paperchromatography. 5-Ketogluconate was not detected in the supernatant of glucose oxidation but sometimes the strain produces

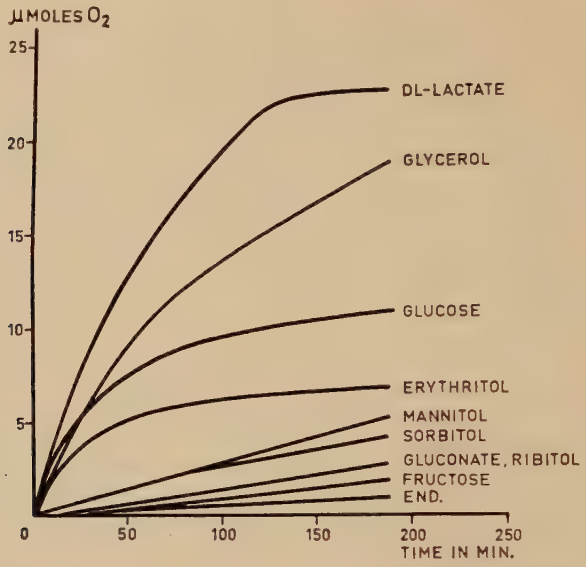

Fig. 7. Oxygen consumption by resting cells of *A. aceti* (Delft). The oxidation of erythritol, sorbitol, mannitol, ribitol and fructose is relatively slow in comparison with the oxidation by the suboxydans strains (fig. 4). The oxidation curve for glucose obtains the same slope as that for gluconate after an O_2 consumption of more than 0.5 mole O_2. This indicates that glucose is partly oxidixed after phosphorylation.

some small Ca-5-ketogluconate crystals on yeast extract-glucose-$CaCO_3$ slants after prolonged incubation. Young cells of this strain oxidized glucose and gluconate with much higher O_2 uptakes than old cells (from 60 to 90% of the amount needed for complete oxidation). Young cells were thus giving the same picture as oxydans strains, which show irisation on glucose-$CaCO_3$.

b. Strains showing irisation on glucose.

A. mesoxydans var. *saccharovorans* oxidizes glucose, gluconate, 2-ketogluconate and 2.5-diketogluconate with uptakes of about 70% of the amount needed for complete oxidation. 5-Ketogluconate was oxidized only in the presence of another oxidizable substrate (fig. 6). It was shown, that in this strain the ketogluconates were metabolized by the same pathway as in *A. suboxydans* (isol. 18). This was not the case however in *Gluconacetobacter liquefaciens*. Glucose, gluconate and 2-ketogluconate were rapidly oxidized to 2.5-diketogluconate, which was oxidized slowly. These compounds were oxidized to the same end products, which are according to AIDA, FUJII and ASAI (1) glycolate, tartronate and three γ-pyrone derivatives. 5-Ketogluconate was metabolized with an O_2 uptake, which was even much higher than that of glucose, indicating that 5-ketogluconate is metabolized by another pathway than glucose, gluconate and 2-ketogluconate.

Cell free extracts of *A. mesoxydans* var. *saccharovorans* and *Gluconacetobacter liquefaciens* were found to contain kinases for glucose, gluconate, glycerol and dihydroxyacetone. G-6-P- and 6-PG-dehydrogenases were also detected. In *A. mesoxydans* var. *saccharovorans* the presence of all the enzymes of the pentose cycle could be demonstrated. The presence of this cycle had been shown previously in a strain of *A. xylinum* by GROMET, SCHRAMM and HESTRIN (12). A tentative interpretation of these results is given in fig. 8.

DISCUSSION.

The strains of the oxydans group are characterized by the presence of an active system for the oxidation of lactate, ethanol, acetate and the dicarboxylic acids, probably the citric acid cycle. A pentose cycle is also present, but the possibility to feed C_6 compounds into the pentose cycle is very limited (fig. 3), but already better developed than in *A. peroxydans* (3, 5, 24).

In the suboxydans group we have more or less the opposite picture. The citric acid cycle is absent, the pentose cycle is the main pathway for oxidation and most strains are able to oxidize a large number of substrates in this way (fig. 5).

The mesoxydans group clearly takes an intermediate position. A citric acid cycle as well as a pentose cycle is present and many substrates can be oxidized (fig. 8).

As was to be expected many strains are borderline cases between two groups. According to the FRATEUR characters they belong in one group but in other properties they seem more related to strains of other groups. Thus *A. rancens* var. *turbidans* (isol. 26) apparently contains hexokinase, mannitoldehydrogenase and 2-ketogluconoreductase which enzymes are normally absent in oxydans strains, but more characteristic of mesoxydans strains.

Again *A. mesoxydans*, though producing 2- and 5-ketogluconate from glucose, does not oxidize mannitol, sorbitol and fructose. This strain also gives a sharp inflection in the respiration curve

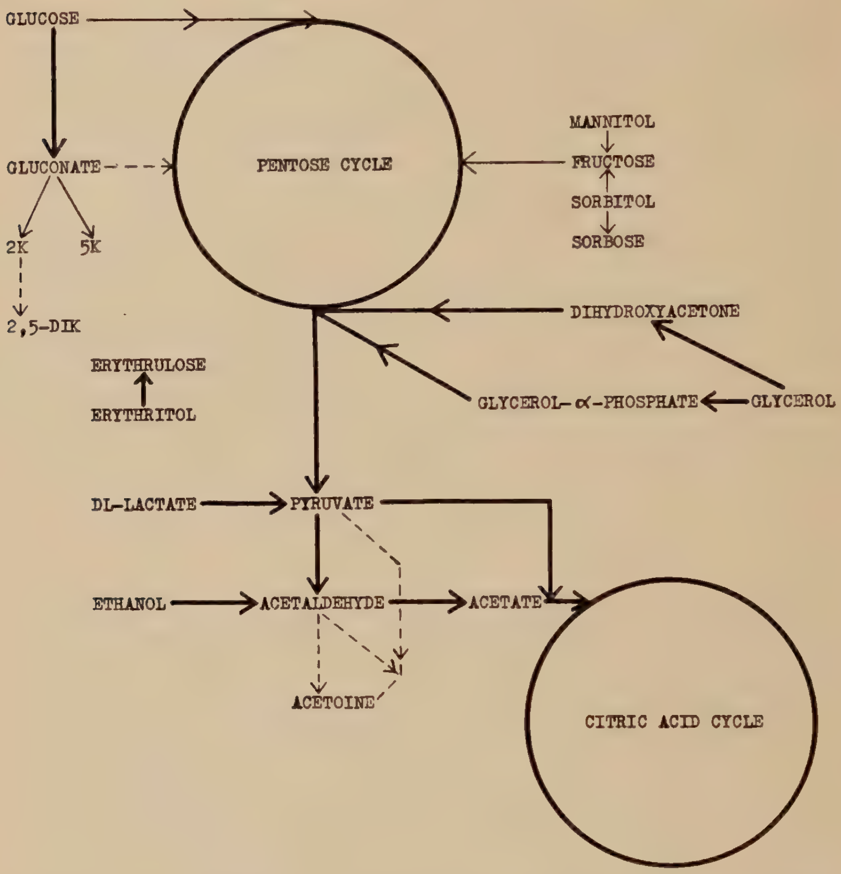

Fig. 8.
Carbohydrate metabolism of *Acetobacter* strains of the mesoxydans group.

for ethanol as is common for oxydans strains. This strain, though belonging in the mesoxydans group has several properties, which one would rather expect in the oxydans group.

A. mesoxydans var. *saccharovorans* takes an intermediate position between the mesoxydans and suboxydans group. It is similar to strains of the suboxydans group because of its behaviour towards ethanol and acetate, which cannot be oxidized completely.

Many more intermediate strains will probably be found. We should bear in mind however that many of the strains in this study were already selected from a large number of isolates because of their aberrant properties:

A. rancens var. *turbidans* (isol. 26) was studied because of its positive oxydogram on mannitol;

A. mesoxydans because of its negative oxydogram on mannitol and sorbitol;

A. suboxydans var. *biourgianum* (isol. 2) because of its negative oxydogram on mannitol.

Among about 50 oxydans strains only 7 were giving irisation on glucose-$CaCO_3$. Four of these were used in the present study. Thus the strains, which can only oxidize glucose to gluconate seem to be more frequent.

Aberrant strains are therefore probably not too common.

The strains with intermediate properties stress the phylogenetic relations between the four *Acetobacter* groups (and the unity of the whole genus). De Ley forwarded the hypothesis that *A. peroxydans* originated in the course of evolution from mesoxydans or oxydans strains by a serie of consecutive loss variations (5). Gromet, Schramm and Hestrin suggested that *A. xylinum* is a phylogenetic ancestor of *A. suboxydans* (12). Thus the *Acetobacter* strains of the mesoxydans group seem to be the ancestors of all other groups. Such a hypothesis would explain why strains with all combinations of properties are found.

Summary.

Twenty *Acetobacter* strains from the oxydans, mesoxydans, and suboxydans group have been studied. Oxygen consumption with a variety of substrates has been determined and various enzymes have been demonstrated in cell free extracts. From the results a tentative picture of carbohydrate metabolism in the three groups of catalase-positive *Acetobacter* strains could be derived (fig. 3, 5, 8). The old concept of the "oxydative power" of various *Acetobacter* groups should be dropped. Instead the properties of *Acetobacter* strains should be discussed in terms of the oxidative pathways involved and the number of substrates that can be fed into these oxidative systems.

The pentose cycle seems to be present in all groups. The citric acid cycle is present in oxydans and mesoxydans strains but absent in the suboxydans group. The number of substrates that can be fed into the pentose cycle increases from peroxydans to suboxydans.

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QUANTITATIVE DETERMINATION OF THE DISSEMINATION OF FLURY RABIES VIRUS IN THE CENTRAL NERVOUS SYSTEM OF THE GUINEA-PIG AFTER INTRAMUSCULAR INOCULATION IN THE HIND LEG

by

C. HUYGELEN and **J. MORTELMANS**

(Received Oktober 7, 1958).

In a previous article (HUYGELEN and MORTELMANS, 1958) we described the typical ascending paralysis, observed in guinea-pigs after inoculation of the Flury strain of rabies virus in the muscles of the hind leg.

The experiments here reported, have been carried out to the purpose of determining whether the propagation of this ascending paralysis corresponds to a proportional increase of the virus titer in the different sections of the nervous system, from the lumbosacral cord to the brain.

The theory of the dissemination of rabies virus by the nervous way is based upon the experiments of DI VESTEA and ZAGARI (1887, 1889) and has been confirmed by GAMALEIA (1887), ROUX (1888, 1889), BARDACH (1888), HELMAN (1889) and BERTARELLI (1904). Several authors (KLIGLER and BERNKOPF, 1943; REMLINGER and BAILLY, 1928, 1929; PALAWANDOW and SEREBRENNAJA, 1931; NICOLAU and KOPCIOWSKA, 1934; NICOLAU, DIMANESCO-NICOLAU and GALLOWAY, 1929) examined the different sections of the central nervous system for the presence of virus during the incubation period of experimental rabies, but, as far as we can ascertain, no quantitative study of the propagation of the virus has been made hitherto.

MATERIALS AND METHODS.

All experiments were carried out on guinea-pigs, weighing between 350 and 400 g, and 1 month old mice, obtained from the breeding colony of the laboratory. We used a Flury virus having undergone 47 egg passages and having a titer of $10^{-3,65}$ determined by intracerebral inoculation of serial tenfold dilutions into mice. The virus was inoculated as a 40%-chick embryo suspension. The intramuscular injection of the guinea-pigs was given in the gastrocnemius muscles.

Suspensions of the different portions of the nervous system and the liver of the guinea-pigs were made in normal saline, and after centrifugation, 0.03 ml of each suspension was injected into mice by the intracerebral route. Generally 4 mice were used for each dilution and each mouse was observed for 21 days. Much attention has been paid to the identical treatment, manipulation and conservation of the different portions of the central nervous system.

EXPERIMENTS.

In our first experiments 6 guinea-pigs were inoculated with 1 ml of Flury virus in the hind legs: 4 out of them have been examined after death and the other two after the onset of paralysis. The skull and the vertebral column of each guinea-pig was opened aseptically and a sample taken from 1) the brain hemispheres, 2) the bulb, 3) the thoracic cord and 4) the lumbosacral cord. After centrifugation, serial tenfold dilutions, ranging from 10^{-1} to 10^{-4}, were injected intracranially into mice.

Guinea-pig n° I died in the night following the 8th day after inoculation and guinea-pig n° VI in the night following the 11th day; in both cases the test material was taken several hours after death. Guinea-pig n° II died in the night following the 9th day and the samples were collected shortly after death. Guinea-pig n° III died in the morning of the 11th day and the material was obtained immediately after death. Both guinea-pig n° IV and guinea-pig n° V were killed in the paralytic stage on the 11th day and the samples were taken immediately afterwards. The results of the mouse inoculations are summarized in table 1. In one case (guinea-pig n° III) no difference could be demonstrated between the titers of the different portions of the central nervous system, because all inoculated mice

TABLE 1.

Mortality ratio of mice, inoculated intracranially with material from guinea-pigs in the paralytic stage and post mortem.

Nº	Lumbosacral cord				Thoracic cord				Bulb				Brain			
	dilution				dilution				dilution				dilution			
	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
guinea-pig																
I	4/4	3/3	3/3	4/4	2/2	4/4	0/3	0/3	3/4	1/4	2/4	0/4	1/4	0/3	0/4	0/4
II	4/4	4/4	4/4	4/4	3/3	4/4	4/4	2/4	3/3	3/3	3/3	4/4	4/4	2/2	4/4	2/4
III	4/4	4/4	4/4	3/3	2/2	3/3	2/2	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4	4/4
IV	4/4	3/3	3/3	3/3	2/4	1/4	0/3	0/3	3/3	4/4	2/4	0/4	4/4	4/4	0/3	0/4
V	4/4	4/4	4/4	4/4	3/3	4/4	2/4	0/4	4/4	2/2	2/4	0/4	4/4	1/4	0/3	0/3
IV	3/3	4/4	3/3	4/4	3/3	3/3	3/4	2/4	3/3	4/4	4/4	3/4	4/4	4/4	3/3	2/4

first number = number of mice which died from rabies.

second number = number of mice inoculated, not included those which died from an intercurrent disease or from cerebral trauma.

succumbed to the infection even at the highest dilution. However, the difference in the length of the incubation period in the mice, having received a 10^{-4} -dilution of the lumbosacral cord, and in those, having received a 10^{-4} -dilution of the brain, indicated that most probably a difference in virus titer would have been obtained, if still higher dilutions had been tested. The average incubation period in the former was 6-7 days, in the latter 13-14 days. The length of the incubation period is generally considered as being directly correlated with the virus concentration (see a.o. NIKOLITSCH, 1957).

In the other five guinea-pigs a quantitative difference could be demonstrated between the mortality ratio of the mice, inoculated with a suspension of the lumbosacral cord, and those, inoculated with a suspension of the brain.

The results of the inoculation of the thoracic cord and the bulb were nearly identical, the bulb showing generally a slightly higher titer.

The titer of the brain did not differ greatly from that of the bulb and thoracic cord, except for guinea-pig n° I: at a dilution of 10^{-1} only one mouse out of four died from rabies. This observation may support the supposition we made previously (HUYGELEN and MORTELMANS, 1958), that, in some cases, after inoculation in the hind leg, guinea-pigs may succumb to Flury rabies infection, before the virus has reached the brain, or more exactly, before the virus has multiplied sufficiently in the brain to be demonstrable by mouse inoculation.

In a second series of experiments we compared the virus content of the two extremities of the central nervous system, *i.e.* the lumbosacral cord and the brain hemispheres, during the incubation period; simultaneously a suspension of the liver of each guinea-pig was injected intracranially into mice.

Each guinea-pig received 2 ml of a 40 %-chick embryo suspension in the gastrocnemius muscles and after 1/2, 1, 2, 4, 6 1/2, 9,24, 72, 96, 120, 144, 168, and 192 hours one guinea-pig was killed and samples taken from the brain hemispheres, the lumbosacral cord and the liver. In view of making the detection of very small amounts of virus possible, we used in each case a highly concentrated suspension (1/5); higher dilutions were also used from the 5th day onwards, *i.e.* about the time a sufficient increase of the virus content was likely to occur. The results are summarized in table 2. Nine out of ten control animals, inoculated with the same virus, died from rabies between the 8th and 13th day after inoculation.

TABLE 2.

Mortality ratio of mice, inoculated intracranially with material obtained from guinea-pigs, killed on various intervals in the incubation period.

Hours after inoculation	Liver	Lumbosacral cord					Brain				
	dilution 1/5	dilution					dilution				
		1/5	10^{-1}	10^{-2}	10^{-3}	10^{-4}	1/5	10^{-1}	10^{-2}	10^{-3}	10^{-4}
$\frac{1}{2}$	0/3	—	—	—	—	—	—	—	—	—	—
1	0/4	0/4	—	—	—	—	0/4	—	—	—	—
2	0/3	0/4	—	—	—	—	0/4	—	—	—	—
4	0/4	0/3	—	—	—	—	0/4	—	—	—	—
$6\frac{1}{2}$	0/4	0/4	—	—	—	—	0/4	—	—	—	—
9	0/4	0/4	—	—	—	—	0/4	—	—	—	—
24	0/3	0/4	—	—	—	—	0/4	—	—	—	—
48	0/4	0/4	—	0/4	—	—	0/4	—	0/4	—	—
72	0/4	1/4	—	0/4	—	—	0/4	—	0/4	—	—
96	0/4	2/4	—	0/4	—	—	0/4	—	0/4	—	—
120	0/3	1/4	0/4	0/4	0/4	0/4	0/4	0/3	0/3	0/4	0/4
144	0/4	3/3	4/4	2/4	1/4	0/4	0/4	0/3	0/4	0/4	0/4
168	0/4	0/3	0/3	0/4	0/4	0/4	0/4	0/3	0/4	0/4	0/4
192	0/4	4/4	3/3	4/4	4/4	4/4	3/4	0/4	0/4	0/4	0/3

first number = number of mice which died from rabies.

second number = number of mice inoculated, not included those which died from an intercurrent disease or from cerebral trauma.

From the 3rd day on virus could be recovered from the lumbosacral cord: one inoculated mouse died and further serial passage proved the Flury virus to be the cause of its death. In the guinea-pigs killed on the 4th and the 5th day, the virus content of the lumbosacral cord was about the same but in the guinea-pig killed on the 6th day (144 hours) a marked increase could be noticed in the lumbosacral cord, whereas from its brain no virus could be recovered.

The results obtained with the guinea-pig killed after 168 hours, at first sight somewhat confusing, may be explained in two ways : 1) the most probable is that this guinea-pig, if it had not been killed, would have survived the infection, since the inoculation of Flury virus, even at the high dosage we used in this experiment, never results in a 100% mortality; in the surviving animals the virus is probably neutralized before it can multiply in the central nervous system; 2) a second possible explanation is that this guinea-pig, if permitted to live, would have shown a long incubation period and that the virus, at the moment the animal was killed, had not yet reached or had not yet multiplied sufficiently in the nervous system to be detectable by mouse inoculation.

From the 8th day on (192 h.) the virus was found not only in the lumbosacral cord, but also in the brain, the concentration in the former however being at least a thousand times higher than in the latter.

The inoculations of liver suspensions only gave negative results.

Discussion.

Although the number of guinea-pigs, which could be examined, as well as the number of mice, used for each virus dilution, were relatively small, the results reported above, are, in our opinion, sufficiently uniform to draw some general conclusions.

These results seem to be in accordance with the theory of the dissemination of rabies virus by the nervous pathway. After intramuscular inoculation in the hind leg the virus is found at first in the lumbosacral cord, where it multiplies and afterwards spreads in the direction of the brain and the animal dies before the virus in the brain has reached as high a titer as in the lumbosacral cord. This propagation of the virus runs parallel with the symptoms of ascending paralysis, observed in guinea-pigs after inoculation of Flury virus in the hind leg. This ascending paralysis is not observed when the virus is injected in the anterior part of the body: in the foreleg or in the masseter muscles.

The fact that no virus could be detected in the liver during the incubation period, does not agree with the observations of KRAUSE (1957). The results however depend greatly upon the quantity and titer of the virus used in the experiments: the LD50 endpoint of our Flury strain, as stated above, was only $10^{-3,65}$, whereas the fixed virus strain used by KRAUSE had a much higher titer, so that very small amounts of virus could still be detected by him in the organs, some time after the inoculation and the subsequent dispersion throughout the experimental animal's body.

The results also demonstrate that during the incubation period the virus content of some parts of the central nervous system may reach a very high titer before causing clinical symptoms : *e.g.* in the guinea-pig, killed after 192 hours, the LD50 point in mice obtained by inoculation of the lumbosacral cord, was at least 10^{-4} (the exact LD50 endpoint being impossible to determine because no further dilutions have been tested); in spite of this considerable amount of virus present in its lumbosacral cord, this guinea-pig, at the moment it was killed, showed no paralysis of the hind legs, nor any other nervous symptoms.

Summary.

A quantitative determination was made of the virus content of the different portions of the central nervous system of guinea-pigs, inoculated with the Flury strain of rabies virus in the muscles of the hind leg. The titration was carried out by intracranial inoculation into mice with material obtained from guinea-pigs killed at various intervals during the incubation period and during the clinical disease and also from guinea-pigs which had succumbed to the infection. The results demonstrate that the virus first multiplies in the lumbosacral cord and afterwards spreads in the direction of the brain. The virus concentration however, remains at any time higher in the lumbosacral cord, being the first to be invaded; this is found during the incubution period, as well as during the paralytic stage and after death.

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ADDITIONS TO THE KNOWN NODULATING SPECIES OF *LEGUMINOSAE*

by

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INTRODUCTION.

Knowledge concerning the occurrence of rhizobial root-nodules on the world range of *Leguminosae* is definitely limited, judging from published summaries (ALLEN and ALLEN 1947; ALLEN and BALDWIN 1954), and refers to at most 15% of all described leguminous species. Available information concerning nodule incidence on legumes indigenous in Australia, summarized by BOWEN (1956), is also very limited. In Western Australia, over 800 species of *Leguminosae* are indigenous, but less than 6% of these are mentioned in BOWEN's summary.

In a study undertaken at this Institute, rhizobial nodules have been observed on some of the species in the subfamilies *Mimosaceae* and *Papilionaceae* occurring indigenous in the plant-geographical South-West Province (GARDNER, 1942) of Western Australia, and on two species in the Eremean Province. Nodulation has not been recorded previously for these species.

NODULATION.

Table 1 presents species of *Leguminosae* on which nodulation has been observed, and which have not been recorded previously as nodulating species. All occur indigenous in the South-West Province of Western Australia, except those of the genus *Swainsona*, which occur in the Eremean Province. Specific epithets and authors are taken from GARDNER (1930).

TABLE 1.

Additions to the known nodulating species of *Leguminosae*.

S. F. MIMOSACEAE

Acacia acuminata Benth.
A. alata R. Br.
A. bidentata Benth.
A. biflora R. Br.
A. Blakelyi Maiden
A. celastrifolia Benth.
A. colletioides (A. Cunn.) Benth.
A. cyanophylla Lindl.
A. cyclopis A. Cunn.
A. diptera Lindl.
A. Drummondii Lindl.
A. eremophila Fitzg.
A. ericifolia Benth.
A. erinacea Benth.
A. extensa Lindl.
A. filifolia Benth.
A. genistoides (F. v. M.) Benth.
A. glaucoptera Benth.
A. Harveyi Benth.
A. hastulata Smith
A. horridula Meissn.
A. Huegelii Benth.
A. microbotrya Benth.
A. Mooreana Fitzg.
A. nervosa D. C.
A. nigricans R. Br.
A. obscura D. C.
A. pentadenia Lindl.
A. pulchella R. Br.
A. restiacea Benth.
A. rostellifera Benth.
A. spathulata (F. v. M.) Benth.
A. squamata Lindl.
A. stenoptera Benth.
A. strigosa Link.
A. subcaerulea Lindl.
A. sulcata R. Br.
A. Tamminensis Pritzel
A. tetragonacarpa Meissn.
A. tetragonophylla F. v. M.
A. urophylla Benth.
A. volubilis F. v. M.

S. F. PAPILIONACEAE

Aotus Preissii Meissn.
A. Tietkinsii F. v. M.
Bossiaea aquifolium Benth.
B. dentata Benth.
B. eriocarpa (R. Br.) Benth.
B. Laidlawiana Tovey et Morris
B. linophylla R. Br.
B. ornata (Lindl.) Benth.
B. Preissii Meissn.
B. pulchella Meissn.
B. Webbii F. v. M.
B. aphyllum Hook.
Brachysema lanceolatum Meissn.
B. latifolium R. Br.
B. praemorsum Meissn.
B. sericeum (Sm.) Domin
Burtonia conferta D. C.
Chorizema aciculare (D.C) C.A. Gardn.
C. cytisoides Turcz.
C. Dicksonii Grah.
C. ericifolium Meissn.
C. reticulatum Meissn.
Daviesia aphylla (F. v. M.) Benth.
D. brevifolia Lindl.
D. cordata Sm.
D. flexuosa Benth.
D. hakeoides Meissn.
D. incrassata Sm.
D. pectinata Lindl.
D. reversifolia F. v. M.
D. teretifolia (R. Br.) Benth.
Dillwynia cinerascens R. Br.
D. divaricata (Turcz.) Benth.
D. uncinata (Turcz.) C.A. Gardn.
Euchilopsis linearis (Benth.) F.v.M.
Eutaxia densifolia Turcz.
E. epacridioides Meissn.
E. obovata (Labill.) C.A. Gardn.
E. virgata Benth.
Gastrolobium Brownii Meissn.
G. crassifolium Benth.
G. obovatum Benth.
G. pulchellum Turcz.
G. spinosum Benth.

G. trilobum Benth.
G. velutinum Lindl.
G. villosum Benth.
Gompholobium amplexicaule Meissn.
G. Knightianum Lindl.
G. marginatum R. Br.
G. polymorphum R. Br.
G. tomentosum Labill.
G. venustum R. Br.
Hardenbergia Comptoniana (Andr.) Benth.
Hovea chorizemifolia (Sweet) D.C.
H. elliptica (Sm.) D.C.
H. pungens Benth.
H. trisperma Benth.
Isotropis cuneifolia (Sm.) Domin
I. juncea Turcz.
Jacksonia floribunda Endl.
J. furcellata (Bonpl.) D.C.
J. hakeoides Meissn.
J. horrida D.C.
J. spinosa (Labill.) R. Br.
J. Sternbergiana Hueg.
J. umbellata Turcz.
Kennedya carinata (Benth.) Domin
K. coccinea Vent.
Kennedya eximia Lindl.
K. nigricans Lindl.
K. Stirlingii Lindl.
Latrobea hirtella (Turcz.) Benth.
Mirbelia dilatata R. Br.
M. floribunda Benth.
M. ovata Meissn.
M. ramulosa (Benth.) C.A. Gardn.
M. spinosa Benth.
Oxylobium atropurpureum Turcz.
O. capitatum Benth.
O. lanceolatum (Vent.) Druce
O. parviflorum Benth.
O. reticulatum Meissn.
Phyllota barbata Benth.
Pultenaea ericifolia Benth.
P. ochreata Meissn.
P. reticulata (Sm.) Benth.
P. strobilifera Meissn.
P. verruculosa Turcz.
Sphaerolobium alatum Benth.
S. grandiflorum (R. Br.) Benth.
S. medium R. Br.
Swainsona microphylla Gray.
S. Incei Price
Templetonia sulcata (Meissn.) Benth.

DISCUSSION.

All observations were made on excavated root systems of field plants, and in examinations over a three year period, a range of nodule condition was recognized. Nodules ranged from turgid, sound, apparently functional structures to withered necrotic remnants. On the exclusively perennial, mostly sclerophyllous native legumes observed in the South-West Province, incidence of sound nodules on nodulating species is not universally constant throughout the year. Forty species in this province were examined during both July (in winter) and March (in summer). Of the 317 individuals examined in July, 94% bore sound nodules. Of the 220 individuals examined in March, 3% bore sound nodules. Unpublished work of HANNON is cited (NORRIS, 1956) as demonstrating the possibility of cyclical nodulation in perennial shrub and tree legumes in New South Wales. A similar phenomenon is suggested in these data.

All sound nodules invariably exhibited in section both a discrete cortex and a central pigmented core, the latter being red or pink in nodules from pre-flowering or flowering hosts. Nodules from *Acacia pulchella, Bossiaea eriocarpa, Chorizema Dicksonii, Gastrolobium spinosum, Hovea chorizemifolia, Isotropis cuneifolia, Kennedya prostrata* and *Oxylobium capitatum* were examined histologically. Tissues were fixed in Nemec fixative (MILOVIDOV, 1928) and were dehydrated and embedded by the tertiary butyl alcohol method (JOHANNSEN, 1940). Milovidov's staining schedule (MILOVIDOV, 1928) was employed. Bacterial shapes were demonstrated in the central tissues of all the nodules in sections cut at 4 microns thickness.

Spherical, cylindrical, branched and corralloid nodule shapes were observed singly or in clusters on tap roots, lateral roots or on both. Nodules were never completely sessile on a major root, but retained vascular contact with the host through a comparatively fragile filament. Many species of native legumes in the South-West Province are exceedingly sclerophyllous, possessing large lignified root-stocks often set in hard substrate. Hand excavation of such root systems and demonstration of nodulation is difficult. Because of this, attempts on many species not included in this list have been ineffectual, and the nodulation status not determined.

Non-rhizobial nodular structures have been observed on the root systems of *Kennedya prostrata, Acacia pulchella, Gastrolobium spinosum* and *Hovea chorizemifolia*. Although distributed on roots in patterns resembling observed rhizobial nodulation patterns on the same species, those nodular structures were distinguishable from rhizobial nodules by the lack of cortex or bacterial zone in their mostly lignified tissues.

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Summary.

Observations have been made on the nodulation of species of *Leguminosae*. Nodulation is recorded for 137 previously unlisted

species in 24 genera, including 8 previously unlisted genera, all species being indigenous in Western Australia.

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(Department of Bacteriology, University of Connecticut, Storrs).

STUDIES OF *MICROCOCCUS CRYOPHILUS* [1]

PART I. EFFECT OF TEMPERATURE AND AERATION ON MULTIPLICATION

by

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and **ROBERT C. CLEVERDON**

(Received March 7, 1959).

1. INTRODUCTION.

For the production of cells of *Micrococcus cryophilus* for metabolic and cytologic studies (to be reported elsewhere), shaking large volumes ($\sim$ 1 l) was unfavorable, although in test tubes, reciprocal agitation yielded markedly improved growth for CLEVERDON (1956) and SHEKOSKY (1958). This cryophilic culture is oxybiontic and fails to grow in Weiss-Spaulding jar and in Spray dish, which suggested a study of the interrelations of agitation, temperature and incubation, and 3 types of culture vessel in use in the laboratory, *viz.*, ,,low form'' flask, Erlenmeyer flask, and test tube. Environmental effects were assessed by plate count (and computation of generation time) or by turbidity.

2. EXPERIMENTAL.

The culture, no. 11052 of the American Type Culture Collection, was maintained at 4⁰ C. in a medium of the following composition in g/l: casitone, 5; yeast extract, 3; NaCl, 5; glucose, 1; K_2HPO_4, 3; pH 7.2; autoclaved at 121⁰ C., 15 psi. This broth was used for all studies. (Broth cultures were viable after 12 months at 4⁰ C.). Cellular suspensions were prepared by diluting a 48 hour shaken test tube culture so that 0.2 ml added to the tempered ,,low form'' flask resulted in a colony count of from 500 to 800/ml at zero time; four loopfuls were added to the Erlenmeyer flask and one loopful to the test tube.

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The agitated cultures were shaken at 110 oscillations/minute; the static cultures were installed on a shelf in the same refrigerated incubator used by CLEVERDON (1956). Temperatures were -2°, 8°, 18°, 28°, ±1° C.

Studies of multiplication in the „low form" flask[1]) at 8°, 18°, 28°, ±1° C. with and without shaking, were made by plate count using the casitone medium above solidified with 1.5% agar. Initial volume of culture medium was 850 ml; surface/volume ratio was 0.6 cm^{-6}. Zero time counts were made after shaking the inoculated vessels for 7 seconds. Thereafter, frequent counts were made in duplicate, using water as a diluent. To assure replicability, it was necessary to maintain the diluent at 18° C., to temper the melted agar at 42° C., and immediately after mixing the inoculum and medium, to cool quickly to 18°C. Plates were incubated at 18° C. for 96 hr, the colonies were counted with a Quebec counter. The numbers were converted to the $\log_2$ for calculation of the generation time following MONOD (1942).

Studies of multiplication in the Erlenmeyer flask and test tube was estimated by turbidity, measured at 580 $m\mu$. The 125 ml Erlenmeyer flasks[2]) used were modified with a side arm insertion into the Coleman Jr. Spectrophotometer; contained 20 ml and were closed with 50 ml beakers. The s/v ratio was 1.6 cm^{-6}. The Kimble 125 × 15 mm test tubes contained 5 ml were closed with linerless bakelite caps; s/v ratio was 0.08 cm^{-1}. The turbidity in tubes was estimated with the Lumetron model 400 A.

3. DISCUSSION.

Culture in „low form" flask (8°, 18°, 28° C.). Typical data in table 1 show that the culture dies at 28° C. and that in the „low form" flask, multiplication is not aided by shaking in the initial period (45 hr at 8° C. and 28.5 hr at 18° C.). Shaking yielded a slightly larger cell crop upon extended incubation; aeration and removal of staling substances may account for this

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[1]) Corning Cat. No. 4422. Corning Glass Works, Corning, N.Y., modified for screw cap closure.

[2]) Cat. No. 14514, Macalaster Bicknell Co., 181 Henry St., New Haven, Conn.

TABLE 1.

Multiplication of *Micrococcus cryophilus* at 8°, 18°, and 28°C., during 147 hours, as shown by plate counts, in „low form" flask with and without agitation; generation times at 8° and 18°C.

Log$_2$ Plate Count/ml								
8°C.			18°C.			28° C.		
Age (hr)	Static	Shaken	Age (hr)	Static	Shaken	Age (hr)	Static	Shaken
0	11.688	11.551	0	9.714	9.022	0	13.070	13.050
1	10.688	11.551	1	9.451	9.344	1	13.019	12.598
2	10.773	11.229	2	9.570	9.409	2	13.070	13.001
3	12.610	12.229	3	9.892	10.103	3	12.688	12.644
4	12.773	12.425	4	10.996	10.399	4	12.372	13.103
5	13.028	12.666	5.5	11.773	10.451	6	12.710	12.503
8	13.288	12.984	13	15.288*	15.392	7	12.288	12.288
9	13.804	13.331	14	15.897	15.773	8	12.136	12.001
10	13.844	13.489	15	16.288	15.897	9	11.688	12.103
11	14.445	14.175	16	16.932	16.409	10	11.103	11.451
22.5	16.861	16.966	16.5	16.932	16.610	11	10.892	11.167
24	17.497	17.064	17	17.185	16.873	22.5	8.229	9.103
26	17.617	17.631	18.5	17.680	17.659	40	0	0
28	18.261	17.920	19	17.932	—	72	0	0
30	18.396	18.497	21	19.219	19.417			
33	18.780*	18.714*	21.5	19.310	—			
36	19.592	20.277	23	20.069	—			
45	22.069	22.632	28.5	22.718*	22.839*			
52.5	22.760*	24.750*	40	25.217	28.311			
97	26.802	30.662	75	28.112	30.150			
121	26.590	30.340	120	29.444	30.662			
147	28.713	30.808						
+G (hr)	4.85	3.18		2.08	1.95			

+G Generation time calculated for intervals denoted in each column by asterisks.

increase. The generation time at 8° C. was shorter in shaken than in static culture, 3.18 *vs* 4.85 hr, whereas at 18° C. virtually no difference was observed, suggesting that at 8° C., shaking promotes multiplication by a factor other than by dissolving oxygen in the medium.

Culture in Erlenmeyer flask (-2°, 8°, 18° C.). Typical data in Table 2 show that as assessed by turbidity measurements, at -2° C. the static flask showed more growth than the shaken flask for the first 247 hr, but thereafter shaking enhanced the growth up to 404 hr. Thus, for this culture at -2° C. in the Erlenmeyer, shaking

TABLE 2.

Multiplication of *Micrococcus cryophilus* at -2°, 8°, and 18°C., during 404 hours in Erlenmeyer flasks, with and without shaking, as assessed by turbidity.

Turbidity								
-2°C.			8°C.			18°C.		
Age (hr)	Static	Shaken	Age (hr)	Static	Shaken	Age (hr)	Static	Shaken
170	10*	0*	57	10*	10*	24	5*	5*
192	10	5	69	20	35	26	5	10
212	20	10	72	25	45	30	5	15
222	25	10	74	30	55	32	5	20
237	35	20	76	30	60	44	10	50
247	40	30	78	40	70	48	25	50
267	45	45	80	45	80	72	45	75
283	50	60	96	50	80	96	55	75
312	60	80	120	60	80	120	60	75
334	65	80	145	70	80	145	65	75
359	70	80	168	75	80			
381	75	80						
404	80	80						

* Figures represent turbidities (100 minus % transmission rounded to nearest 5) using the Coleman Jr. Spectrophotometer at 580 mμ.

aided growth only after a high population was reached, suggesting the removal of staling substances, or early over-aeration. At 8° C. and 18° C. however, shaking always aided growth and the time required to attain maximum turbidity was markedly shortened.

Culture in test tubes (-2°, 8°, 18° C.). Typical data in Table 3 show that agitation of test tube cultures at -2° C. resulted in improved growth only after 283 hr, suggesting over-aeration. At 8° C., improvement in growth by shaking was first observed in 72 hr; at 18° C., in 30 hr. The value of shaking is especially observed in test tubes at 18° C. where the s/v ratio is smallest.

Summary.

Micrococcus cryophilus, while oxybiontic, grows better if shaken in air only if temperature and shape of vessel are conducive. All studies revealed that shaking ultimately leads to a larger cell crop, the effect being most prominent at 18° C. in test tubes. At -2° C., in Erlenmeyer flasks, agitation appeared clearly detrimental in early

TABLE 3.

Multiplication of *Micrococcus cryophilus* at -2ºC., 8ºC., and 18ºC., during 404 hr, in test tubes, with and without shaking, as assessed by turbidity.

Turbidity								
-2ºC.			8ºC.			18ºC.		
Age (hr)	Static	Shaken	Age (hr)	Static	Shaken	Age (hr)	Static	Shaken
170	10*	10*	57	10*	10*	24	5*	5*
192	15	10	69	15	20	26	10	15
212	15	15	72	15	20	30	15	40
222	20	20	74	15	20	32	20	60
237	20	20	76	15	25	44	35	80
247	25	25	78	15	30	48	40	80
267	25	25	80	25	35	72	55	80
283	35	45	96	30	50	96	70	80
312	35	45	120	35	65	120	70	80
334	40	50	145	40	80	145	70	80
359	45	60	168	50	80			
381	45	65						
404	50	70						

* Figures represent turbidities (100 minus % transmission, rounded to nearest 5) using the Lumetron 400A at 580 mμ.

growth. The generation time of *M. cryophilus* in the „low form" flask at 18º C. was about 2.0 hr whether culture was shaken or static; at 8º C., generation times were 4.85 hr when static and 3.18 hr when shaken. For production of cells, incubation at 18º C. is ideal, shaken if in tube or Erlenmeyer flask, static if in „low form" flask.

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INTERMEDIATES OF THE ENDOGENOUS RESPIRATION OF YEAST

by

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(Received April 24, 1959).

INTRODUCTION.

In preceding experiments, using the phenylhydrazine trapping technique, SCEVOLA and BOLCATO (1952) were able to isolate from the endogenous respiration of yeast the triose phosphates, pyruvic acid and small amounts of acetaldehyde. Continuing those experiments with the same technique, we have now succeeded in isolating the successive intermediates of the oxidation of stored polysaccharide i.e. the acetyl group, glyoxylate, formaldehyde and the formyl group, which are also the intermediates of our monocarboxylic acid scheme (MAS) of the direct oxidation of acetate (BOLCATO *et al.*, 1957; BOLCATO, 1959a). With preliminary experiments we have observed that phenylhydrazine, like 2.4-dinitrophenol (ROTHSTEIN and BERKE, 1952; STICKLAND, 1956), made the attack on stored polysaccharide of yeast possible. The results of these experiments are reported and discussed in the present report.

METHODS.

Procedures. A single brand of commercial pressed baker's yeast was used (Clastidium). 30 g fresh wt. was suspended in 1000 ml water. The pH was adjusted with dilute sulphuric acid to 5.2 and the suspension aerated in 1500 ml cylindrical glass tube through a sintered-glass disk at the base of the tube. The phenylhydrazine oxalate (PO) was added to the cell suspension as indicated in table 2-A. More reproducible results and greater yields of formaldehyde were obtained in the experiments with dimedon along with phenylhydrazine: table 2-B (Bolcato *et al.*, 1956a). To test whether phenylhydrazine alone (in the form of PO salt) could induce, like 2,4-dinitrophenol (DNP), a consumption of the stored polysaccharide, determinations were made of the polysaccharide content of the yeast cells before and after 10 hrs of aeration in the tube described above. The results of these preliminary experiments, resumed in table 1, clearly demonstrate that phenylhydrazine had the same effect as the DNP on the oxidation of carbohydrate reserves. This permitted us to operate with phenylhydrazine alone, this performing the dual function of promoting the oxidation of these reserves and of trapping the intermediates of the process. In fact, in some experiments in the presence of both phenylhydrazine and DNP, no particular qualitative difference could be observed in the trapped intermediates. Moreover, in the presence of the two substances there was noted a greater consumption of the stored carbohydrate, i.e. 37.8 per cent of the total carbohydrate instead of 31.3 per cent and 27.95 per cent respectively in the presence of 4 mM of PO salt and 3 mM of DNP.

TABLE 1.

Consumption of stored polysaccharide of yeast during 10 hrs of aeration. (The conditions of the experiments are given in the text. Data mean of 3 experiments).

Treatment of the yeast	Polysaccharide content mg/g dry wt		Consumption per cent of total polysaccharide
	initial	final	
without any addition	198	165	13.15
With 3 mM of DNP	198	145	27.95
With 4 mM of PO	198	136	31.30
With 3 mM of DNP and 4 mM of PO	198	123	37.80

Isolation of the intermediates. *β*-acetylphenylhydrazide (Bolcato *et al.*, 1952), acetophenone and benzaldehyde (Bolcato and Scevola, 1953), glyoxylic acid (Bolcato *et al.*, 1956b) and formaldehyde (Bolcato *et al.*, 1956a; Bolcato *et al.*, 1956b) were isolated according to the methods previously reported. It is only to be kept in mind that acetophenone and benzaldehyde are respectively the results of the acetylation and formylation of the benzene ring of the phenylhydrazine (Bolcato and Scevola, 1953; Bolcato *et al.*, 1956c) and thus the expression of the formation, during the oxidative process, of the acetyl and the formyl groups. The polysaccharide content of the yeast, before and after aeration, was determined according to the method used by Stickland (1956). The value obtained (total material capable of yielding reducing sugar on acid hydrolysis) will be referred to as "polysaccharide" and will be expressed as the equivalent amount of glucose. Care was taken to avoid any error due to evaporation, during aeration, by measuring the initial and final volume of the yeast cell suspension.

TABLE 2.

Endogenous oxidation of stored polysaccharide of yeast in presence of phenylhydrazine oxalate (PO) alone or in presence of PO with dimedon (for experimental conditions see text).

Time	pH	PO mg/l added	Acetyl[1] mg/l	Glyoxylic acid mg/l	Formaldehyde mg/l	Formyl[1] mg/l
A: with PO alone						
10.00	5.2	0.7	—	—	—	—
10.50	5.2	0.3	—	—	—	—
12.40	5.3	0.2	—	—	—	—
15.00	5.3	—	—	—	0.5	—
17.00	5.3	—	—	1.2	—	—
19.00	5.3	—	12.0	0.7	0.0	3.5
B: with PO and dimedon (dimedon g 1/l)						
11.40	5.2	0.6	—	—	0.0	—
12.40	5.2	0.6	—	—	0.0	—
13.40	5.2	0.3	—	—	traces	—
14.40	5.2	—	—	—	1.0	—
16.50	5.2	—	—	—	1.5	—
17.30	5.3	—	—	—	1.8	—
18.30	5.3	—	—	—	2.8	—

[1] The acetyl group is calculated from the isolated β-acetylphenylhydrazide and acetophenone and the formyl group is calculated from the isolated benzaldehyde.

Results and Discussion.

The data reported in table 2 permit the conclusion that a possible way of oxidation of the polysaccharide reserves of yeast may be the MAS, all the intermediates of the system being isolated during the present experiments. This conclusion is independent of the possible participation of the tricarboxylic acid cycle, evidence of which in the cells our technique does not allow. As we have reported in the preceding paper (SCEVOLA and BOLCATO, 1952), the precursors of the acetyl group, from which the oxidation of the polysaccharide reserves starts, are pyruvic acid and triose phosphates. Thus, the oxidation of the polysaccharide of yeast can be summarized by the following series of reactions:

polysaccharide → triose phosphates → pyruvate → acetyl → glycolate → glyoxylate → formaldehyde → formyl → $H_2O + CO_2$

Our experiments do not establish whether the triose phosphates are formed by the Embden-Meyerhof pathway or by that of the hexose monophosphate. As can be seen in table 2 only small amounts of glyoxylic acid and formaldehyde could be trapped in the presence of phenylhydrazine alone. This is due to the fact that the acid and the aldehyde are oxidized, also in the form of their phenylhydrazones, with the intermediate formation of the formyl group in the form of the carbonyl carbon of benzaldehyde (BOLCATO *et al.*, 1956c; BOLCATO, 1959b). On the other hand a good yield of formaldehyde was obtained with the simultaneous presence of phenylhydrazine and dimedon (Table 2-B). As we have reported in recent papers, glyoxylate cannot arise from reactions connected with the malic synthetase or the isocitritase (BOLCATO 1959a; BOLCATO, 1959b). The amounts of the intermediates trapped from the endogenous respiration are always lower than those obtained from the acetate oxidation, the greater part of the phenylhydrazine being fixed from the triosephosphates and from pyruvic acid (SCEVOLA and BOLCATO, 1952).

Summary.

The intermediates trapped from experiments on the endogenous respiration of yeast cells, in the presence of phenylhydrazine, suggest that a possible way of oxidation of the stored polysaccharide may be the following:

polysaccharide → triose phosphates → pyruvate → acetyl → glycolate → glyoxylate → formaldehyde → formyl → $H_2O + CO_2$

It has been also observed that the phenylhydrazine, like 2.4-

dinitrophenol, stimulates the endogenous consumption of the polysaccharide reserves of yeast.

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(From the National Institute of Public Health, Utrecht, The Netherlands, and the Communicable Disease Center, Public Health Service, U. S. Department of Health, Education and Welfare, Atlanta, Georgia, U.S.A.).

A NEW *SALMONELLA* TYPE: *SALMONELLA UTRECHT*

by

E. H. KAMPELMACHER, MARY A. FIFE and **P. R. EDWARDS**

(Received March 19, 1959).

Salmonella utrecht was received from Dr D. A. A. Mossel (Central Institute for Nutritional Research, Utrecht) who isolated the organism from fish meal from Portuguese Angola. *S. orion* (3, 10: y: 1,5) also was present in the culture and the two types were separated by colony selection.

S. utrecht possessed cultural and biochemical characteristics typical of the *Salmonella* group. The organism produced hydrogen sulfide, failed to form indol, was methyl red positive and Voges-Proskauer negative. It failed to hydrolyze urea, to grow in KCN medium, or to liquefy gelatin. Nitrates were reduced and the lysine decarboxylase reaction was positive. Prompt growth occurred in Simmons' citrate medium and d-tartrate medium was promptly acidified. Acid and gas were produced promptly from glucose, arabinose, rhamnose, xylose, maltose, trehalose, mannitol, dulcitol, and sorbitol. Lactose, sucrose, adonitol, inositol, glycerol, and salicin were not fermented.

Upon serologic examination it was found that *S. utrecht* was not agglutinated by any of the sera representative of the recognized *Salmonella* O groups. It was tested with sera prepared from all recognized Arizona and *E. coli* O groups and was agglutinated only by serum for Arizona O group 31, with which it reacted to titre. An O antiserum prepared from *S. utrecht* likewise agglutinated Arizona O 31 antigen to titre. Reciprocal absorption tests resulted in complete removal of agglutinins from both sera. The O antigens of *S. utrecht* were assigned the symbol 52.

The H antigens of *S. utrecht* were diphasic and phase 1 was flocculated to one-half the titre of *S. typhi* H serum and absorption tests reduced the titre of the serum by 50 per cent. Phase 2 was agglutinated to the titre of *S. thompson* phase 2 serum and in absorption tests reduced the titre of the serum from 1-20,000 to 1-1,000. The absorbed serum no longer agglutinated phase 2 of *S. panama* but did agglutinate phase 2 of *S. muenster* at 1-200. The H antigens of phase 1 and phase 2 of *S. utrecht* were designated as d and 1,5 respectively.

Summary.

A new *Salmonella* type isolated from Angola fish meal is called *S. utrecht*. The organism possesses an O antigen not hitherto recognized in the *Salmonella* group to which the symbol 52 is assigned. The O antigen is identical with that of Arizona O group 31. The antigenic formula of *S. utrecht* is 52: d: 1,5.

(From the Communicable Disease Center, Enteric Bacteriology Unit, Public Health Service, U.S. Department of Health, Education and Welfare, Atlanta, Georgia, U.S.A.).

ON ANTIGENIC O-RELATIONSHIPS BETWEEN THE GROUPS *SALMONELLA*, *ARIZONA*, *ESCHERICHIA* AND *SHIGELLA* ¹)

by

E. H. KAMPELMACHER ²)

(Received June 5, 1959).

Antigenic relationships between the different groups of the Family *Enterobacteriaceae* are frequently observed and described by many authors. KAUFFMANN (1954) gives an extensive review concerning studies of different workers in this field. In addition to these publications in which particular relationships are described, systematic research in this field was carried out by KAUFFMANN (1954), EDWARDS and EWING (1955), EWING *et al.* (1956) and FRANTZEN (1950).

KAUFFMANN (1954) examined the *Salmonella* O-antigens 1—30 and *Escherichia* strains representing 60 different O groups. In recent years EDWARDS (1955) reported on the extensive O relationships between the groups *Salmonella* and *Arizona* and EWING (1956) on relationships between the O groups of *Escherichia* and *Shigella*. FRANTZEN (1950) extended research further with the *Salmonella* O-antigens 1—39, the *E. coli* antigens 1—110 and the *Arizona* O-antigens 1—25.

Since these investigations new O-antigens were discovered in the different groups and it was desirable to carry out a systematic

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¹) This work has been performed during a stay in the Enteric Bacteriology Unit of the Communicable Disease Centre, Atlanta (Ga.), U.S.A. It was sponsored by the Fulbright Organisation and the Department of Social Affairs and Public Health, The Netherlands.

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research on present known O-antigens of the above mentioned groups.

In the following work the relationships between the *Salmonella* O-antigens 1—52, the *Arizona* O-antigens 1, 2—1, 33, the *E. coli* O-antigens 1—142 and OX 1-OX 13 and the *Shigella* O-antigens A 1-A 10 (subgroup *S. dysenteriae*), B 1-B 6 (subgroup *S. flexneri*), C 1-C 15 (subgroup *S. boydii*) and D (subgroup *S. sonnei*), were studied.

MATERIALS AND METHODS.

Strains of the different groups of *Enterobacteriaceae* as received for routine identification and typing in the Enteric Bacteriology Unit of the Communicable Disease Center, Atlanta, were used.

For the preparation of antigens single smooth colonies of the different O-group strains were isolated from nutrient agar plates as used for bacteriophage typing and inoculated in horse infusion broth. After 12—18 hours incubation (37°C.) the broth cultures of *Salmonella*, *Arizona* and *Shigella* were heated at 100°C. for 1 hour. Broth cultures of *Escherichia* were heated at 100°C. for 1½ hours. After heating formalin was added in amount of 0.3%. If necessary these antigens were diluted with 0.5% formalinized saline to obtain uniform turbidity.

Anti-serums prepared in rabbits from special strains held for this purpose in above mentioned laboratory were used. For the first screening antigens were tested with different serums mixed in pools. The grouping of anti-serums in these pools are shown in Table 1 (p. 291).

All agglutination reactions were carried out in dilutions ranging from 1 : 100 to 1 : 6400. The tubes were placed in a waterbath by 45°C. for 18—24 hours. In case of a positive reaction in one of the pool serums agglutination reactions with all single serums of this pool were performed.

RESULTS.

A. Agglutination tests.

a) Relationships between *Salmonella* O-antigens and anti-serums of the groups *Salmonella*, *Arizona*, *Escherichia* and *Shigella*.

In table 2 (p. 292-294) are included the positive agglutination reactions obtained with *Salmonella* O-antigens by serums of the *Salmonella*, *Arizona*, *Escherichia* and *Shigella* groups.

TABLE 1.

Grouping of anti-serums in pools.

Pools	I	II	III	IV	V	VI	VII	VIII
Salmonella	1, 2, 12 1, 4, 5, 12 9, 12 4, 12, 27 8 (8), 20 9, 46	6, 7 6, 14, 24 1, 6, 14, 25 18	3, 10 3, 15 (3), (15), 34 1, 3, 19 11 41	1, 13, 22, 36 1, 13, 23, 37 (1), 49	16 17 21 28 30	35 38 39 40 42 43	44 45 47ab 47ac 48 50 51 52	
Arizona 1)	1, 2 1, 3 1, 4 1, 33	6 8 9ab 9ac 10ab 10ac 11	7ab 7ac 13 17 27	5 14 28 29	12 15 16 17 18	19 20 21 22 23	24 25 26 30 31 32	
Shigella	A 1 A 2 A 3 A 4 A 5	A 6 A 7 A 8 A 9 A 10	B 1a B 1b B 2a B 2b B 6	B 3a B 3c B 4a B 4b B 5	C 1 C 2 C 3 C 4 C 5	C 6 C 7 C 8 C 9 C 10	C 11 C 12 C 13 C 14 C 15	D
Escherichia 2)	1 2 3 4 5 6 7 18 23 25 26 38 50 64 65 70 71 74 102 114 116 138 139	8 9 10 11 12 13 14 15 16 17 19 60 73 77 125 129 133 135	20 21 22 24 27 28ab 28ac 29 30 32 33 34 35 36 37 42 48 76 81 83 85	39 40 41 43 44 45 46 49 51 52 53 54 55 56 57 59 66 106 108 118	58 61 62 63 68 69 75 78 79 80 82 84 86 90 92 124 126 127 128	87 88 89 91 93 95 96 97 98 99 100 101 103 104 105 107 109 115 117	110 111 112 113 119 120 121 123 130 131 132 134 136 137 140 141	OX 1 OX 2 OX 3 OX 4 OX 5 OX 6 OX 7 OX 8 OX 9 OX 10 OX 11 OX 12 OX 13

1) grouping as used by EDWARDS. 2) grouping as used by EWING.

b) Relationships between *Arizona* O-antigens and anti-serums of the groups *Arizona*, *Salmonella*, *Escherichia* and *Shigella*.

In table 3 (p. 295) are included the positive agglutination reactions obtained with *Arizona* O-antigens by serums of the *Arizona*, *Salmonella*, *Escherichia* and *Shigella* groups.

c) Relationships between *Escherichia* O-antigens and anti-serums of the groups *Escherichia*, *Salmonella* and *Arizona*. (The results of O-antigen relationships between *Escherichia* and *Shigella* groups may be found in the recent work of EWING and coworkers).

text continues p. 294

TABLE 2.

Relationships between *Salmonella* O antigens and the antisera of the *Salmonella, Arizona, Escherichia* and *Shigella* groups.

Salmonella antigen	*Salmonella* serum	Titre	*Arizona* serum	Titre	*E. coli* serum	Titre	*Shigella* serum	Titre
2, 12	1, 2, 12	6400	6	400				
	9, 12	800	16	200				
			23	200				
1, 2, 12	1, 2, 12	1600						
	1, 4, 5, 12	800						
	13, 23	200						
1, 4, 5, 12	1, 4, 5, 12	6400						
	1, 2, 12	800						
	4, 12, 27	200						
4, 12	4, 12, 27	400						
	1, 2, 12	200						
	1, 4, 5, 12	800						
1, 4, 12, 27	4, 12, 27	3200						
	1, 2, 12	200						
	1, 4, 5, 12	1600						
	8	400						
	(8), 20	400						
	3, 10	400						
	3, 15	200						
	13, 22	400						
	13, 23	400						
	(1), 49	400						
4, 5, 12	1, 4, 5, 12	3200						
	1, 2, 12	200						
1, 4, 12	1, 2, 12	800						
	1, 4, 5, 12	800						
	1, 3, 19	400						
	13, 23	100						
8	8	>6400						
	(8), 20	1600						
8, 20	(8), 20	6400						
	8	1600						
	18	100						
9, 12	9, 12	3200	6	400				
	1, 2, 12	400	7ab	100				
	1, 4, 5, 12	1600	16	200				
	(9), 46	200	19	100				
	(3), (15), 34	400						
1, 9, 12	9, 12	800						
	1, 2, 12	800						
	1, 4, 5, 12	400						
	(3), (15), 34	400						
	1, 3, 19	400						
	13, 23	200						
6, 7	6, 7	400	27	400	62	100		
	6, 14, 24	200			68	200		
	6, 14, 25	200						
6, 8	8	400	27	400	73	200		
	6, 7	200	32	200	62	200		
	6, 14, 24	800						
3, 10	3, 10	>6400						
	3, 15	1600						
	(3), (15), 34	800						
	1, 3, 19	800						
3, 15	3, 15	6400						
	3, 10	1600						
	(3), (15), 34	1600						
	1, 3, 19	800						

Table 2 (continued).

Salmonella antigen	*Salmonella* serum	Titre	*Arizona*-serum	Titre	*E. coli* serum	Titre	*Shigella* serum	Titre
(3), (15), 34	(3), (15), 34	3200			68	200	A 3	100
	3, 10	800					B 2a	400
	3, 15	400					B 2b	200
	1, 3, 19	100						
	1, 4, 5, 12	400						
	1, 6, 14, 25	200						
1, 3, 19	1, 3, 19	3200			17	200	B 1a	100
	3, 10	3200			73	100	B 1b	200
	3, 15	3200			106	400	B 2a	400
	(3), (15), 34	1600					B 2b	200
	1, 2, 12	1600					B 4b	100
	1, 4, 5, 12	1600						
	(9), 46	400						
11	11	3200	17	1600	75	800		
13, 22	13, 22	1600	1, 2	400	127	400		
	13, 23	1600	18	1600				
1, 13, 23	1, 13, 23	3200	18	3200	127	800		
	13, 22	800						
6, 14, 24	6, 14, 24	6400	7ab	400	73	800		
	6, 14, 25	1600	7ac	3200	77	800		
	6, 7	400	32	1600	66	400		
	18	1600						
	1, 2, 12	400						
6, 14, 25	6, 14, 25	800			17	800	B 2a	100
	6, 14, 24	400			77	200	B 2b	100
	1, 2, 12	400			106	800		
	1, 4, 5, 12	200			62	200		
					68	200		
16	16	6400	25	1600				
17	17	3200	12	800	85	400	B 1a	400
							B 1b	100
							C 3	200
18	18	800	7ab	200				
	6, 14, 24	200						
	6, 14, 25	100						
21	21	3200	22	800				
28	28	400			5	100		
					70	200		
					62	400		
30	30	3200	10ab	400	71	200		
	40	100						
35	35	800			111	400		
38	38	800	1, 2	200	21	400		
			16	400				
39	39	800			OX9	100		
40	40	800	10ab	400	6	200		
	(1), 49	400	10ac	200				
	30	100						
1, 40	40	800	10ab	800	6	800		
	1, 2, 12	200	10ac	800				
	1, 4, 5, 12	200						
	13, 22	200						
	13, 23	200						
	(1), 49	800						
41	41	1600	13	400				
	6, 7	100	27	100				
42	42	3200	15	800	1	1600	A 1	200
	47ab	100			64	800		
43	43	1600			86	100		
	13, 23	400						
44	44	1600	1, 2	3200				
	38	100	1, 3	1600				

Table 2 (continued).

Salmonella antigen	*Salmonella* serum	Titre	*Arizona* serum	Titre	*E. coli* serum	Titre	*Shigella* serum	Titre
45	45	1600	9ac	200				
	50	200	11	800				
9, 46	9, 46	800						
	9, 12	800						
47ab	47ab	3200	1, 2	200				
	47ac	200	23	1600				
47ac	47ac	1600	28	400	118	400		
	48	100	29	400				
48	48	1600	5	800				
			29	100				
(1), 49	(1), 49	400	10ac	400	6	100		
50	50	1600	9ab	400	55	1600		
			9ac	3200				
51	51	800	1, 2	3200	23	400		
	44	400	9ab	400				
52	52	6400	31	3200	7	400	C 12	100
					62	800		
					107	1600		

In table 4 (p. 296-309) are included the positive agglutination reactions obtained with *Escherichia* O-antigens by serums of the *Escherichia*, *Salmonella* and *Arizona* groups.

As an extract from table 4 table 4a (p. 310) shows the O-antigen relationships between *E. coli* serotypes reported in diarrheal disease and the *Salmonella* and *Arizona* groups.

d) Relations between *Shigella* O-antigens and anti-serums of the groups *Shigella*, *Salmonella* and *Arizona*. (The results of O-antigen relationships between *Escherichia* and *Shigella* groups may be found in the recent work of Ewing and coworkers (1956)).

In table 5 (p. 311-313) are included the positive agglutination reactions obtained with *Shigella* O-antigens by serums of the *Shigella*, *Salmonella* and *Arizona* groups.

B. Absorption tests.

In case of a positive agglutination result in both directions cross-absorptions were performed in the usual way. The same antigens as prepared for the agglutination tests were used for the agglutinations with the absorbed serums.

a) O-antigen relationships between the *Salmonella* and *Arizona* groups.

In table 6 (p. 314-316) a survey of the results of tube agglutination tests before and after absorption of the different serums is given. In case of identity the antigen numbers are underlined.

text continues p. 309

TABLE 3.

Relationships between *Arizona* O-antigens and the anti-serums of the *Arizona, Salmonella, Escherichia* and *Shigella* groups.

Arizona antigen	*Arizona* serum	Titre	*Salmonella* serum	Titre	*E. coli* serum	Titre	*Shigella* serum	Titre
1, 2	1, 2	3200	44	200	23	200		
	1, 3	100	51	400				
1, 3	1, 3	1600	44	1600				
	1, 2	1600	51	200				
1, 4	1, 4	800			62	100		
5	5	1600	48	1600				
	29	200						
6	6	1600			35	400	B 2a	200
7ab	7ab	800	18	1600				
7ac	7ac	400	6, 14, 24	400	77	200		
			6, 14, 25	200				
			18	100				
8	8	1600			2	400		
					50	400		
					74	400		
9ab	9ab	3200	47ab	200	55	1600		
	9ac	6400	50	3200				
9ac	9ac	3200	50	1600	55	800		
	9ab	400						
10ab	10ab	1600	(1), 49	800				
	10ac	400						
10ac	10ac	400	(1), 49	400				
11	11	1600	45	200	OX13	400		
12	12	800	17	1600			C 3	100
13	13	800	41	1600				
14	14	3200	(1), 49	100			A 7	1600
	5	800	43	100				
15	15	800	42	3200	1	800		
16	16	400	38	800	21	100		
17	17	1600	11	1600	75	800		
	1, 2	100						
18	18	800	13, 22	200	127	100		
			13, 23	1600				
19	19	400						
20	20	400	35	800	111	200		
21	21	400	43	400	86	200		
22	22	400	21	1600				
23	23	800	47ab	800				
24	24	800						
	27	400						
25	25	800	16	800				
26	26	1600						
27	27	1600	6, 7	800			C 12	100
	7ab	400	6, 14, 24	400				
	7ac	400	6, 14, 25	400				
			18	1600				
			41	400				
28	28	800	47ac	800	118	400		
	29	400	48	200				
29	29	800						
	5	100						
30	30	1600			78	800		
	25	200						
31	31	800	52	3200			C 12	100
32	32	800	6, 14, 24	1600	73	400		
	7ac	200	6, 14, 25	200	68	200		
1, 33	1, 33	1600			12	400		
	1, 2	400			123	400		

TABLE 4.

Relationships between *Escherichia* O-antigens and the anti-serums of the *Escherichia*, *Salmonella* and *Arizona* groups.

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
1	1	6400	(8) 20	100	6	800
	2	100	42	1600	15	800
	5	200				
	50	200				
	64	400				
	OX 11	200				
	OX 12	100				
2	2	1600			6	800
	1	800			8	200
	50	1600				
	71	400				
	95	800				
	117	400				
	OX 11	100				
3	3	3200	1, 4, 5, 12	400	1, 2	800
	4	400	(8), 20	400		
	22	200	6, 7	100		
	23	400	6, 14, 24	400		
	26	100	6, 14, 25	100		
	70	200	(3), (15), 34	400		
			13, 22	400		
			17	800		
			28	400		
			44	800		
			45	100		
			47ab	100		
			51	200		
4	4	6400	(8), 20	200	9ab	100
	12	400	16	200	10ac	100
	16	200	47ac	200	28	200
	18	1600			25	100
	25	400				
	26	100				
	62	400				
	68	800				
	73	100				
	102	800				
	125	100				
	129	100				
	133	100				
5	5	3200	13, 23	400	10ab	800
	7	800	28	3200		
	65	3200	39	400		
	70	>6400	40	200		
	71	3200	45	400		
	112	200				
	114	800				
6	6	3200	1, 4, 5, 12	200	1, 2	200
	54	100	(1), 49	>6400	1, 33	100
	57	400	40	1600	6	200
					10ab	400
					10ac	1600
7	7	6400	28	800	14	100
	5	400	30	100	31	200
	25	200				
	36	100				
	51	200				
	65	800				
	68	800				

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	70	400				
	71	3200				
	116	400				
	141	100				
8	8	3200	6, 7	400	27	100
	43	200				
	46	3200				
	60	800				
	119	200				
9	9	1600				
10	10	3200	42	200	6	400
	1	200			9ab	800
	100	800			15	100
	OX 10	400				
11	11	3200	3, 10	400	7ac	100
	4	200	16	3200	25	1600
	36	200	39	400		
	125	100	40	400		
12	12	3200	6, 7	100	1, 2	1600
	4	200	11	100	1, 33	1600
	25	200	16	100	17	200
	123	400	21	200	5	200
	OX 1	200	38	400		
			39	200		
			47ab	100		
			48	200		
13	13	3200	1, 4, 5, 12	100	1, 2	400
	3	400	(8), 20	400	6	200
	4	800	6, 7	400	5	100
	17	100	6, 14, 24	800	30	400
	18	800	6, 14, 25	400	32	100
	19	400	(3), (15), 34	200		
	23	800	13, 22	800		
	35	200	(1), 49	200		
	44	400	28	200		
	45	200	48	400		
	50	400				
	53	200				
	59	400				
	62	800				
	68	1600				
	70	200				
	73	800				
	99	100				
	102	800				
	106	200				
	125	400				
	129	800				
	133	400				
	135	800				
	138	100				
	139	200				
	140	200				
14	14	1600	9,12	200		
	93	200				
	OX 13	100				
15	15	1600	47ac	200	5	800
	36	400	48	800	19	1600
	43	100			28	200
	45	800				
16	16	3200	6, 7	200	1, 4	200
	12	200			1, 33	400

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	13	100			9ab	400
	58	200				
	62	3200				
	125	800				
	129	800				
	135	1600				
17	17	1600	1, 2, 12	800	8	200
	62	1600	1, 4, 5, 12	400	7ab	800
	68	1600	(8), 20	100	7ac	1600
	73	400	6, 7	200		
	77	1600	6, 14, 24	3200		
	106	1600	6, 14, 25	3200		
	135	200	18	1600		
			1, 3, 19	800		
			13, 22	400		
18	18	3200	18	100	1, 2	800
	4	400	16	100		
	13	100	44	400		
	25	800				
	62	200				
	68	800				
	102	400				
	117	100				
	126	100				
	133	100				
	135	100				
19	19	1600	13, 23	400	6	200
	5	100	21	100	18	400
	7	100	50	400		
	13	800				
	25	200				
	35	100				
	48	1600				
	50	100				
	68	400				
	86	1600				
	102	400				
	113	800				
	129	200				
	133	800				
	135	800				
20	20	3200				
	OX 10	6400				
21	21	1600	35	800	1, 2	800
	30	400	38	3200	16	1600
	32	200				
	83	100				
	OX 1	200				
	OX 3	800				
22	22	1600	3, 15	100	1, 2	1600
	53	100	50	400	1, 4	400
	55	1600			9ab	400
	76	3200			9ac	1600
	83	800			15	100
	OX 7	100				
23	23	3200	1, 4, 5, 12	100	1, 2	> 6400
	3	400	(8), 20	100	1, 3	400
	6	100	6, 14, 25	400	9ab	1600
	28ab	100	18	400	18	100
	38	3200	(3), (15), 34	200		
	68	800	13, 22	100		
	70	800	13, 23	200		

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	73	200	(1), 49	800		
	112	100	38	800		
	117	200	44	3200		
	OX 1	200	47ab	200		
			51	3200		
24	24	800			16	100
	33	400				
25	25	3200	16	400		
	4	400				
	7	200				
	18	200				
	36	100				
	68	1600				
	102	800				
	120	200				
	138	800				
26	26	3200				
	4	100				
	13	100				
	18	200				
	25	400				
	68	800				
	79	200				
	102	800				
	117	100				
27	27	3200				
28ab	28ab	3200	(1), 49	200	1, 2	200
	28ac	800			1, 4	800
					5	200
					9ab	800
29	29	1600	1, 2, 12	1600		
			1, 4, 5, 12	1600		
			1, 3, 19	400		
			13, 23	800		
			(1), 49	1600		
30	30	3200	38	1600	1, 2	800
	OX 3	200	44	200		
31	synonim with O-antigen 1					
32	32	1600	38	800		
	21	400				
	83	1600				
33	33	3200				
	37	400				
	100	200				
34	34	3200	(8), 20	100	12	400
	13	200	17	800		
	62	1600	28	100		
	68	100	47ab	100		
	69	100				
	73	200				
	85	800				
	132	200				
	140	1600				
35	35	3200			6	>6400
	13	400			7ac	400
36	36	3200	30	100		
	4	400	39	200		
	7	200	48	800		
	25	200				
	26	100				
	62	100				
	[illegible]	1600				

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	102	800				
	120	100				
	141	800				
37	37	3200	21	400	9ab	200
	81	400				
38	38	3200	44	400	1, 2	3200
	23	200	47ab	100	1, 3	400
	OX 1	100	51	800		
39	39	1600	1, 2, 12	100	6	800
	46	200	47ab	100	9ab	100
	49	200	47ac	100	12	100
					16	100
					23	200
					27	400
					28	200
40	40	1600	11	100	1, 4	800
	15	200	44	100	9ab	200
	62	3200			17	400
	74	200			18	200
	75	1600			19	400
	81	800				
	127	400				
	OX 1	100				
	OX 10	100				
41	41	1600	45	1600	11	800
	4	100	47ac	200		
	29	200				
	OX 13	1600				
42	42	3200			10ac	200
	28	400				
	OX 1	100				
43	43	3200	16	400		
	36	800	39	800		
			47ac	400		
44	44	1600	1, 1, 12	200	7ab	400
	68	1600	6,7	400	7ac	1600
	73	400	6, 14, 24	800	32	400
	77	800	6, 14, 25	800		
	106	1600	18	100		
			13, 22	200		
45	45	3200	4, 12, 27	100		
	66	200	(9), 46	200		
			18	200		
			(3), (15), 34	100		
			28	200		
46	46	> 6400	16	100		
	27	400				
	45	200				
	57	400				
	60	100				
	83	800				
	86	100				
	92	100				
	102	100				
	113	400				
	126	100				
	130	200				
	131	400				
	134	800				
	OX 6	800				
47	synonim with O-antigen 105					

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
48	48	1600				
	19	200				
	54	400				
	86	200				
	87	100				
	119	400				
	OX 1	100				
49	49	3200			1, 4	800
50	50	6400			6	800
	1	800			8	>6400
	2	6400				
	5	100				
	74	3200				
	95	400				
	117	100				
51	51	>6400			1, 2	1600
	3	100				
	17	100				
	23	100				
	30	400				
	64	100				
	69	400				
	81	1600				
52	52	>6400	17	200		
	87	200				
53	53	3200	44	800	1, 2	200
	1	100			6	200
	2	200				
	4	200				
	13	100				
	50	200				
	62	400				
	117	100				
	120	100				
	140	400				
54	54	6400				
	20	100				
	35	400				
	48	400				
	59	400				
	120	100				
	OX 10	100				
55	55	6400	47ab	100	9ab	800
			50	>6400	9ac	>6400
56	56	800	1, 2, 12	400	12	200
	46	100	1, 4, 5, 12	100	16	800
	57	100	6, 7	200		
			13, 22	200		
			13, 23	100		
			21	100		
			47ab	400		
			47ac	200		
57	57	1600	47ab	100	12	100
			47ac	100	16	200
58	58	1600	48	200	30	100
					10ac	200
59	59	3200				
	48	800				
	54	400				
	57	800				
	OX 1	200				
	OX 7	200				

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
60	60	>6400				
	8	800				
	39	100				
	73	800				
	91	100				
	93	100				
61	61	3200			26	200
	7	100				
	76	100				
	108	100				
	113	200				
62	62	1600	6, 7	100	1, 2	3200
	5	100	21	400	1, 3	200
	12	800	38	800	1, 33	1600
	13	100	44	800	6	400
	16	800	47ab	200	9ab	400
	23	100	51	200	22	100
	38	200				
	96	200				
	101	100				
	116	200				
	123	800				
	129	400				
	135	800				
	OX 1	800				
	OX 3	100				
63	63	6400				
	OX 1	200				
	OX 10	800				
64	64	6400	42	200	15	100
	1	100				
	27	200				
65	65	3200				
	7	100				
	70	100				
	71	1600				
66	66	>6400	6,7	200	7ac	400
	39	100	6, 14, 24	100	13	200
	45	400	6, 14, 25	400		
	60	100	18	1600		
			41	800		
68	68	3200	(8), 20	100	6	800
	1	1600	21	100		
	4	100				
	25	800				
	62	100				
	71	100				
	129	400				
	138	400				
69	69	>6400	42	100		
	13	200				
	120	100				
70	70	6400	28	1600		
	5	3200	39	200		
	65	1600				
	71	400				
	114	100				
71	71	6400	13, 23	200		
	5	1600	28	1600		
	7	800				
	65	3200				
	70	400				
	117	400				

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	OX 12	100				
73	73	6400	1, 2, 12	400	32	1600
	13	100	1, 4, 5, 12	200		
	17	400	(8), 20	400		
	44	400	6, 7	800		
	60	200	6, 14, 24	3200		
	62	200	6, 14, 25	1600		
	68	6400	1, 3, 19	200		
	78	100	13, 22	400		
	106	800	13, 23	400		
	125	800	35	200		
			45	400		
74	74	6400			8	1600
	2	1600				
	5	100				
	50	1600				
	64	100				
	65	200				
	70	400				
	113	400				
75	75	3200	6, 7	200	1, 2	200
	126	200	6, 14, 25	100	1, 4	400
			3, 10	800	17	3200
			3, 15	400	27	100
			(3), (15), 34	400	28	200
			1, 3, 19	400	25	100
			11	>6400	30	200
			16	400		
			41	200		
76	76	6400			1, 4	400
	22	800			11	800
	87	200				
	101	200				
	117	100				
	130	100				
	OX 13	1600				
77	77	1600	(8), 20	100	8	1600
	17	800	6, 7	800	7ab	800
	73	100	6, 14, 24	3200	7ac	3200
			6, 14, 25	3200	27	200
			18	1600	32	100
78	78	>6400	47ab	100	25	1600
	92	800			30	3200
	118	400				
79	79	>6400				
	25	100				
	68	1600				
	80	1600				
	102	100				
	138	200				
80	80	>6400	18	400	9ab	200
			45	800	9ac	3200
			50	1600	11	100
81	81	>6400				
	37	400				
	51	100				
82	82	3200				
83	83	>6400	38	1600	1, 2	3200
	21	800			16	100
	22	1600				
	32	1600				
	46	200				

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	79	800				
	113	100				
	131	200				
	OX 7	800				
84	84	6400			5	100
	4	200				
	25	200				
	71	200				
	88	400				
85	85	1600	17	1600	12	800
	34	200				
86	86	1600	13, 22	400	18	400
	90	400	13, 23	400	21	400
	127	400	43	1600		
87	87	3200	1, 2, 12	200	6	400
	58	100	1, 4, 5, 12	200	9ab	400
	78	400	6, 7	200	16	800
	88	100	6, 14, 25	100	17	100
	92	100	3, 10	200	27	200
	95	200	47ab	400		
	96	800	47ac	100		
	103	200	48	100		
	104	800				
	131	800				
	140	200				
	OX 1	400				
	OX 13	200				
88	88	>6400			30	400
	141	200				
89	89	1600	11	100		
90	90	1600	13, 22	100	9ac	100
	55	100	13, 23	800	18	400
	86	400	50	100		
	113	200				
	127	800				
91	91	>6400	39	100		
	95	400				
92	92	3200	47ab	200	30	800
	78	800	48	400		
	104	200				
	107	800				
93	93	1600	(8), 20	200	13	100
	8	400	6, 7	100	17	200
	89	800	6, 14, 25	100	27	800
			3, 10	400		
			(3), (15), 34	400		
			41	800		
95	95	>6400				
	103	100				
	OX 1	400				
	OX 9	1600				
96	96	6400				
	78	200				
	105	200				
	120	800				
	OX 1	1600				
97	97	6400	(8), 20	100	6	100
	OX 1	400	(1), 49	200	7ac	100
			47ab	200	9ab	100
					9ac	100
					16	100
					27	100

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
98	98	>6400				
99	99	>6400	(8), 20	800		
	3	100	6, 7	400		
	4	100	6, 14, 25	100		
	13	400	13, 22	400		
	73	1600	28	400		
	87	200				
	108	200				
	125	100				
	129	400				
100	100	800	6, 7	200	1, 2	1600
	10	100	6, 14, 24	200	1, 3	800
	12	200	6, 14, 25	100	1, 33	400
	101	100	18	1600	9ab	6400
			11	200	9ac	800
			38	400	12	100
					15	400
					18	200
101	101	>6400	1, 4, 5, 12	400	6	400
	35	100	(1), 49	800		
	76	800	21	800		
	117	800				
102	102	>6400	(8), 20	100	14	400
	4	1600	3, 10	800	19	100
	7	400	3, 15	200	25	200
	18	800	45	400		
	25	1600	47ab	100		
	26	800				
	36	400				
	71	400				
	107	400				
	120	1600				
	130	1600				
	139	800				
	140	800				
	141	800				
103	103	6400	8	400	5	800
	1	100				
	64	400				
	70	1600				
	74	100				
	87	800				
	95	1600				
	104	200				
	116	800				
	OX 9	1600				
104	104	3200				
	112	100				
105	105	>6400	11	400	1, 2	100
	54	800			6	1600
	115	>6400			9ab	200
	120	1600				
106	106	1600	6, 14, 24	800	7ac	400
	44	800	6, 14, 25	1600		
	68	100				
	73	100				
	77	200				
107	107	1600	47ab	400	31	800
	56	400	47ac	100		
	87	400	48	200		
	89	100				
	101	400				

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	103	200				
	104	100				
	117	3200				
108	108	3200	47ab	400	26	400
	53	800				
109	109	6400	1, 2, 12	1600	7ac	100
	87	800	1, 4, 5, 12	400	15	800
	107	100	47ab	200	16	100
			48	400		
110	110	3200			9ab	800
	87	100				
111ab	111ab	3200	1, 2, 12	200	20	400
			35	1600		
111ac	111ac	6400	35	1600	20	800
112ab	112ab	>6400	18	1600	1, 2	800
	113	800				
	123	100				
112ac	112ac	3200			1, 2	800
	113	200				
	119	200				
113	113	3200	13, 23	400	18	200
	112	400				
114	114	6400	28	400	14	400
	5	800				
	70	200				
	71	200				
115	115	>6400			15	400
	89	100				
	105	800				
	120	400				
116	116	6400	30	1600	1, 33	200
	7	3200			6	100
	64	200			8	100
	65	400				
	70	400				
	71	200				
	78	400				
	101	200				
117	117	6400	21	800	1, 2	100
	26	100	40	400	10ab	200
	49	100				
	50	800				
	71	100				
	113	800				
	120	200				
	129	400				
118	118	1600	47ab	400	5	400
	43	200	47ac	3200	28	3200
	107	100	48	800	29	800
119	119	3200	18	400		
120	120	>6400				
	1	200				
	17	100				
	20	200				
	25	200				
	51	100				
	96	100				
	102	400				
	105	1600				
	115	800				
	133	200				
	139	200				

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	140	200				
121	121	1600			5	400
	123	200			14	800
123	123	1600	21	400	1, 2	3200
	12	3200	44	200	1, 33	>6400
	38	200				
	116	1600				
124	124	6400	(3), (15), 34	1600	1, 2	400
	69	800	47ab	200		
	93	100				
	99	100				
125ab	125ab	>6400	1, 2, 12	200	32	200
	16	100	(8), 20	200		
	36	100	6, 7	1600		
	37	100	6, 14, 24	800		
	68	1600	6, 14, 25	800		
	73	800	28	200		
	113	400	39	400		
			45	400		
125ac	125ac	1600	16	800	25	400
	7	200	28	400		
	11	400	39	400		
	37	200				
	71	200				
	113	400				
126	126	1600	6, 7	100	17	200
	75	800	11	400	27	200
			18	400		
127	127	1600	13, 22	1600	9ac	100
	86	100	13, 23	1600	18	800
	90	800	50	100		
128ab	128ab	1600	13, 23	200	9ab	200
	48	800	45	400	9ac	1600
	81	100	47ab	100	11	200
	87	800	50	1600	18	200
	127	400				
128ac	128ac	800	1, 4, 5, 12	400	8	400
	62	100	(8), 20	200	9ab	100
	63	100	6, 14, 25	200	9ac	800
	127	400	1, 3, 19	200	10ac	100
			13, 22	400	11	400
			13, 23	200		
			45	400		
			47ab	100		
			50	400		
129	129	>6400	(8), 20	200	9ab	200
	4	100	48	200		
	13	1600				
	16	200				
	26	800				
	62	400				
	68	400				
	73	200				
	133	200				
	135	100				
130	130	3200	3, 10	200		
131	131	1600	28	100		
	113	1600				
	125	800				

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	129	100				
	OX 1	400				
132	132	3200			1, 4	400
	16	100				
	51	100				
	62	800				
	118	100				
	140	800				
	OX 3	100				
133	133	6400			6	800
	4	100				
	13	1600				
	18	200				
	19	3200				
	25	800				
	50	200				
	68	1600				
	69	200				
	102	800				
	120	100				
	129	800				
	135	1600				
	138	400				
	OX 4	400				
134	134	1600				
135	135	800	1, 2, 12	200		
	13	200	1, 4, 5, 12	200		
	117	400				
	129	200				
	133	100				
136	136	6400				
	61	800				
	OX 1	200				
	OX 3	100				
137	137	800			30	100
	62	200				
138	138	1600			6	800
	25	400				
	68	1600				
139	139	3200			6	100
	25	400				
	62	100				
	68	1600				
	102	1600				
	133	400				
	135	100				
	138	100				
	OX 5	100				
140	140	>6400	17	1600	8	400
	87	400			12	400
	120	200			16	100
	OX 1	200				
	OX 13	100				
141	141	400				
142	no serum available		47ab	200	1, 2	800
	4	100			6	800
	102	200				
OX 1	OX 1	3200			25	200
	96	800				
OX 2	OX 2	3200				
	27	200				
	76	800				

Table 4 (continued).

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	115	1600				
	OX 13	1600				
OX 3	OX 3	>6400	38	800	16	400
	76	800	47ab	200		
OX 4	OX 4	>6400				
	71	400				
OX 5	OX 5	>6400	1, 2, 12	100	5	400
	11	800	16	800	25	800
	83	400	47ab	100	29	800
	95	400	48	200		
	OX 1	200				
OX 6	OX 6	3200	21	100	5	400
			47ab	200		
			48	1600		
OX 7	OX 7	>6400	6, 14, 25	200		
	83	800				
OX 8	OX 8	1600	6, 14, 25	100	1, 2	400
	11	100	18	400	18	100
	127	200	13, 22	400		
	OX 7	400	13, 23	200		
			21	800		
			44	100		
OX 9	OX 9	>6400	8	200	8	1600
	2	100	39	3200	24	100
	10	100				
	36	200				
	50	800				
	74	400				
	91	100				
	95	1600				
	103	1600				
	125	3200				

b) O-antigen relationships between the groups *Salmonella* and *Escherichia*. In table 7 (p. 317-319) a survey of the results of tube agglutination tests before and after absorption of the different serums is given. In case of identity the antigen numbers are underlined.

c) O-antigen relationships between the groups *Arizona* and *Escherichia*. In table 8 (p. 320-321) a survey of the results of tube agglutination tests before and after absorption of the different serums is given. In case of identity the antigen numbers are underlined.

d) O-antigen relationships between the groups *Salmonella* and *Shigella*. In table 9 (p. 322) a survey of the results of tube agglutination tests before and after absorption of the different serums is given.

e) O-antigen relationships between the groups *Arizona* and *Shigella*. In table 10 (p. 322) a survey of the results of tube agglutination tests before and after absorption of the different serums is given.

text continues p. 316

TABLE 4a.

Relationships between O-antigens of *E. coli* serotypes reported in diarrheal disease and the *Salmonella* and *Arizona* groups.

E. coli antigen	*E. coli* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
26	26	3200				
	4	100				
	13	100				
	18	200				
	25	400				
	68	800				
	79	200				
	102	800				
	117	100				
55	55	6400	47ab	100	9ab *)	800
			50 *)	>6400	9ac *)	>6400
86	86	1600	13, 22	400	18	400
	90	400	13, 23	400	21 *)	400
	127	400	43 *)	1600		
111ab	111ab	3200	1, 2, 12	200	20 *)	400
			35	1600		
112ac	112ac	3200	18	1600	1, 2	800
	113	200				
	123	100				
119	119	3200	18	400		
125ab	125ab	6400	1, 2, 12	200	32	200
	16	100	(8), 20	200		
	36	100	6, 7	1600		
	37	100	6, 14, 24	800		
	68	1600	6, 14, 25	800		
	73	800	28	200		
	113	400	39	400		
			45	400		
125ac	125ac	1600	16	800	25	400
	7	200	28	400		
	11	400	39	400		
	37	200				
	71	200				
	113	400				
126	126	1600	6, 7	100	17	200
	75	800	11	400	27	200
			18	400		
127	127	1600	13, 22 *)	1600	9ac	100
	86	100	13, 23 *)	1600	18 *)	800
	90	800	50	100		
128ab	128ab	1600	13, 23	200	9ab	200
	48	800	45	400	9ac	1600
	81	100	47ab	100	11	200
	87	800	50	2600	18	200
	127	400				
128ac	128ac	800	1, 4, 5, 12	400	8	400
	62	100	(8), 20	200	9ab	100
	63	100	6, 14, 25	200	9ac	800
	127	400	1, 3, 19	200	10ac	100
			13, 22	400	11	400
			13, 23	200		
			45	400		
			47ab	100		
			50	400		

*) = reciprocal

TABLE 5.

Relationships between *Shigella* O-antigens and the anti-serums of the *Shigella, Salmonella* and *Arizona* groups.

Shigella antigen	*Shigella* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
A 1	A 1	>6400	42	400	6	800
	B 1a	200	47ab	200	7ab	100
	B 2a	200	48	200	7ac	400
	B 2b	400	(1), 49	200	8	100
	B 4a	200	50	400	9ab	200
	B 4b	800			10ac	100
	B 5	200			12	100
	C 3	200			15	800
	C 11	100				
	C 12	800				
A 2	A 2	1600	1, 2, 12	800	5	400
	A 1	200	1, 4, 5, 12	200	16	800
	A 10	800	9, 12	800	18	200
	B 1a	200	47ab	800		
	B 1b	200	47ac	800		
	B 2a	400	48	400		
	B 2b	400	52	800		
	B 4b	800				
	C 11	400				
	C 12	400				
	C 15	400				
	D	400				
A 3	A 3	>6400	1, 2, 12	400	1, 2	800
	A 5	800	(8), 20	400	12	400
	D	200	52	1600	16	800
A 4	A 4	3200	47ab	100	29	800
			47ac	100		
A 5	A 5	>6400	47ab	100	30	400
			48	800		
A 6	A 6	>6400	3, 10	1600	1, 4	800
					25	200
A 7	A 7	>6400	13, 23	800	5	800
			43	400	14	1600
A 8	A 8	>6400	3, 15	400	1, 2	3200
			21	800	1, 3	200
			35	400	1, 33	400
			38	800	9ab	400
			44	1600	16	800
			47ab	400		
			51	1600		
			52	400		
A 9	A 9	3200				
A 10	A 10	3200				
	A 2	400				
	C 1	200				
B 1a	B 1a	1600	(8), 20	800	6	800
	B 1b	3200	6, 14, 25	100	10ac	1600
	B 2a	400	13, 22	200	30	400
	B 2b	800	35	100		
	B 3a	800	(1), 49	100		
	B 4a	400				
	B 4b	800				
	B 5	100				
	B 6	800				
B 1b	B 1b	>6400	(8), 20	800	6	100
	B 1a	1600	6, 7	100	10ac	1600
	B 2a	400	6, 14, 25	100	30	100
	B 2b	800	13, 22	200		
	B 3a	3200	(1), 49	100		

Table 5 (continued).

Shigella antigen	*Shigella* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	B 3c	800	35	100		
	B 4a	200				
	B 4b	1600				
	B 5	100				
	B 6	800				
B 2a	B 2a	3200	48	100	1, 4	200
	B 1a	100			6	200
	B 1b	400				
	B 2b	800				
	B 3a	100				
	B 4a	200				
	B 4b	100				
	B 6	400				
B 2b	B 2b	3200	(8), 20	200	9ab	200
	B 1a	100				
	B 1b	200				
	B 2a	1600				
	B 3a	400				
	B 4a	200				
	B 5	400				
B 3a	B 3a	3200				
	B 1b	3200				
	B 2a	100				
	B 2b	800				
	B 3c	400				
	B 4a	200				
	B 4b	1600				
	B 5	100				
B 3b	no serum available					
	B 1a	100				
	B 1b	3200				
	B 2b	1600				
	B 3a	3200				
	B 3c	800				
	B 4a	400				
	B 4b	1600				
	B 5	200				
B 3c	B 3c	1600	48	100	6	100
	B 1a	100				
	B 1b	6400				
	B 2a	400				
	B 2b	200				
	B 3a	6400				
	B 4a	400				
	B 4b	3200				
	B 5	100				
	B 6	400				
B 4a	B 4a	1600	1, 2, 12	400	1, 4	200
	B 1a	200	1, 4, 5, 12	400	6	800
	B 1b	400	1, 3, 19	100	32	400
	B 2a	800	13, 22	100		
	B 2b	400	13, 23	100		
	B 3a	200				
	B 4b	1600				
	B 5	200				
B 4b	B 4b	3200	1, 2, 12	400	6	100
	B 1b	3200	1, 4, 5, 12	400	12	100
	B 2a	400				
	B 2b	100				
	B 3a	3200				

Table 5 (continued).

Shigella antigen	*Shigella* serum	Titre	*Salmonella* serum	Titre	*Arizona* serum	Titre
	B 3c	800				
	B 4a	400				
	B 5	100				
B 5	B 5	1600	(8), 20	100		
	B 1a	100				
	B 2b	1600				
	B 3a	100				
	B 4a	400				
B 6	B 6	>6400	17	200	6	400
	B 1a	1600	28	200		
	B 1b	1600	(1), 49	400		
	B 2a	800				
	B 2b	800				
	B 3a	100				
	B 3c	400				
	B 4a	400				
	B 4b	100				
C 1	C 1	>6400	44	200	1, 2	200
	A 2	400	47ab	200		
	A 10	400				
	C 4	100				
C 2	C 2	3200				
	B 1b	200				
C 3	C 3	>6400	1, 2, 12	200	1, 2	400
	C 11	200	6, 7	400	1, 3	200
	D	200	6, 14, 24	400	16	400
			6, 14, 25	800	27	200
			17	400		
			18	200		
			44	200		
			47ab	100		
			48	400		
			(1), 49	200		
C 4	C 4	1600	44	1600	1, 2	400
	C 1	1600	51	100	6	200
	C 11	800				
C 5	C 5	3200				
C 6	C 6	3200	1, 2, 12	100	11	1600
	C 10	400	6, 7	200	16	100
	D	100	8	100	25	200
			45	100		
			47ab	100		
C 7	C 7	3200				
C 8	C 8	6400				
C 9	C 9	6400	21	100		
	B 4a	200				
	C 13	400				
C 10	C 10	3200	52	400		
	C 6	800				
	C 11	400				
C 11	C 11	1600	11	200	6	400
	C 10	400				
C 12	C 12	1600				
C 13	C 13	800				
C 14	C 14	3200	35	100		
			38	1600		
			52	200		
C 15	C 15	1600	18	800	1, 2	800
	A 2	200	44	100		
	A 8	100	47ab	100		
D	D	3200				

TABLE 6.

O-antigen relationships between the groups *Salmonella* and *Arizona*.

O antiserums	Absorbed by	Titres of O-antigen suspensions	
		S* 44	A** 1, 2
S 44		1600	200
A 1, 2		1600	1600
S 44	A 1, 2	800	< 100
A 1, 2	S 44	< 100	800
		S 51	A 1, 2
S 51		800	400
A 1, 2		3200	1600
S 51	A 1, 2	< 100	< 100
A 1, 2	S 51	< 100	< 100
		S 44	A 1, 3
S 44		1600	1600
A 1, 3		800	800
S 44	A 1, 3	< 100	< 100
A 1, 3	S 44	< 100	< 100
		S 48	A 5
S 48		1600	800
A 5		800	1600
S 48	A 5	400	< 100
A 5	S 48	< 100	200
		S 6, 14, 24	A 7ac
S 6, 14, 24		3200	1600
A 7ac		400	400
S 6, 14, 24	A 7ac	100	< 100
A 7ac	S 6, 14, 24	< 100	< 100
		S 18	A 7ab
S 18		800	200
A 7ab		800	800
S 18	A 7ab	< 100	< 100
A 7ab	S 18	< 100	< 100
		S 50	A 9ab
S 50		1600	400
A 9ab		3200	3200
S 50	A 9ab	800	< 100
A 9ab	S 50	< 100	400
		S 50	A 9ac
S 50		1600	1600
A 9ac		1600	3200
S 50	A 9ac	< 100	< 100
A 9ac	S 50	< 100	< 100
		S (1), 49	A 10ac
S (1), 49		400	400
A 10ac		400	400
S (1), 49	A 10 ac	200	< 100
A 10ac	S (1), 49	< 100	100
		S 45	A 11
S 45		1600	800
A 11		200	1600
S 45	A 11	800	< 100
A 11	S 45	< 100	400

*) S = *Salmonella*. **) A = *Arizona*.

Table 6 (continued).

O antiserums	Absorbed by	Titres of O-antigen suspensions	
		S 17	A 12
S 17		3200	800
A 12		1600	800
S 17	A 12	1600	< 100
A 12	S 17	< 100	< 100
		S 41	A 13
S 41		1600	400
A 13		1600	800
S 41	A 13	100	< 100
A 13	S 41	< 100	< 100
		S 42	A 15
S 42		3200	800
A 15		3200	800
S 42	A 15	100	< 100
A 15	S 42	< 100	< 100
		S 38	A 16
S 38		800	400
A 16		800	400
S 38	A 16	< 100	< 100
A 16	S 38	< 100	< 100
		S 11	A 17
S 11		3200	1600
A 17		1600	1600
S 11	A 17	< 100	< 100
A 17	S 11	< 100	< 100
		S 13, 22	A 18
S 13, 22		1600	1600
A 18		200	800
S 13, 22	A 18	800	< 100
A 18	S 13, 22	< 100	200
		S 13, 23	A 18
S 13, 23		3200	3200
A 18		1600	800
S 13, 23	A 18	200	< 100
A 18	S 13, 23	< 100	100
		S 21	A 22
S 21		3200	800
A 22		1600	400
S 21	A 22	< 100	< 100
A 22	S 21	< 100	< 100
		S 16	A 25
S 16		6400	1600
A 25		800	800
S 16	A 25	< 100	< 100
A 25	S 16	< 100	< 100
		S 6, 7	A 27
S 6, 7		400	400
A 27		800	1600
S 6, 7	A 27	200	< 100
A 27	S 6, 7	< 100	< 100

Table 6 (continued).

O antiserums	Absorbed by	Titres of O-antigen suspensions	
		S 47 ac	A 28
S 47ac		1600	400
A 28		800	800
S 47ac	A 28	400	< 100
A 28	S 47ac	< 100	200
		S 52	A 31
S 52		6400	3200
A 31		3200	800
S 52	A 31	< 100	< 100
A 31	S 52	< 100	< 100
		S 6, 14, 24	A 32
S 6, 14, 24		6400	1600
A 32		1600	800
S 6, 14, 24	A 32	100	< 100
A 32	S 6, 14, 24	< 100	< 100
		S 47ab	A 23
S 47ab		3200	1600
A 23		800	800
S 47ab	A 23	< 100	< 100
A 23	S 47ab	< 100	< 100
		S 35	A 20
S 35		800	400
A 20		800	400
S 35	A 20	< 100	< 100
A 20	S 35	< 100	< 100
		S 40	A 10ab
S 40		800	400
A 10ab		400	1600
S 40	A 10ab	400	< 100
A 10ab	S 40	< 100	200
		S 40	A 10ac
S 40		800	200
A 10ac		400	400
S 40	A 10ac	400	< 100
A 10ac	S 40	< 100	200

Table 11 (p. 322) finally shows the O-antigen of the groups *Salmonella*, *Arizona* and *Escherichia*, which are identical as shown by the absorption tests described in the preceding paragraphs.

Discussion.

The results here reported correspond in general with the findings of Kauffmann, Edwards, Ewing and Frantzen. The investigation, extended further by using the O-antigens found in recent years, shows that the general picture of O-antigen relationships within the Family *Enterobacteriaceae* has not changed. There is a

text continues p. 319

TABLE 7.

O-antigen relationships between the groups *Salmonella* and *Escherichia*.

O antiserums	Absorbed by	Titres of O-antigen suspensions	
		S 42	C*1
S 42		3200	1600
C 1		1600	6400
S 42	C 1	1600	< 100
C 1	S 42	< 100	800
		S 28	C 5
S 28		400	3200
C 5		100	3200
S 28	C 5	200	< 100
C 5	S 28	< 100	800
		S 40	C 6
S 40		800	1600
C 6		200	3200
S 40	C 6	800	< 100
C 6	S 40	< 100	1600
		S 1, 40	C 6
S 1, 40		3200	800
C 6		800	3200
S 1, 40	C 6	800	< 100
C 6	S 1, 40	< 100	800
		S (1), 49	C 6
S (1), 49		400	> 6400
C 6		100	3200
S (1), 49	C 6	200	< 100
C 6	S (1), 49	< 100	400
		S 1, 3, 19	C 17
S 1, 3, 19		3200	200
C 17		800	1600
S 1, 3, 19	C 17	1600	< 100
C 17	S 1, 3, 19	< 100	800
		S 6, 14, 24	C 17
S 6, 14, 24		6400	400
C 17		3200	1600
S 6, 14, 24	C 17	800	< 100
C 17	S 6, 14, 24	< 100	1600
		S 1, 6, 14, 25	C 17
S 1, 6, 14, 25		800	800
C 17		1600	1600
S 1, 6, 14, 25	C 17	100	< 100
C 17	S 1, 6, 14, 25	< 100	400
		S 38	C 21
S 38		800	3200
C 21		400	1600
S 38	C 21	400	< 100
C 21	S 38	< 100	800
		S 51	C 23
S 51		800	3200
C 23		400	1600
S 51	C 23	200	< 100
C 23	S 51	< 100	400

* C = *E. coli*

Table 7 (continued).

O-antiserums	Absorbed by	Titres of O-antigen suspensions	
		S 50	C 55
S 50		1600	1600
C 55		> 6400	6400
S 50	C 55	100	< 100
C 55	S 50	< 100	< 100
		S 6, 7	C 62
S 6, 7		400	100
C 62		100	1600
S 6, 7	C 62	200	< 100
C 62	S 6, 7	< 100	800
		S 42	C 64
S 42		3200	800
C 64		200	> 6400
S 42	C 64	800	< 100
C 64	S 42	< 100	1600
		S 6, 14, 24	C 66
S 6, 14, 24		6400	400
C 66		100	> 6400
S 6, 14, 24	C 66	400	< 100
C 66	S 6, 14, 24	< 100	3200
		S 28	C 70
S 28		400	200
C 70		1600	6400
S 28	C 70	200	< 100
C 70	S 28	< 100	100
		S 6, 7	C 73
S 6, 7		400	200
C 73		800	6400
S 6, 7	C 73	200	< 100
C 73	S 6, 7	< 100	3200
		S 6, 14, 24	C 73
S 6, 14, 24		6400	800
C 73		3200	6400
S 6, 14, 24	C 73	400	< 100
C 73	S 6, 14, 24	< 100	800
		S 45	C 73
S 45		1600	100
C 73		400	6400
S 45	C 73	800	< 100
C 73	S 45	< 100	1600
		S 11	C 75
S 11		3200	800
C 75		> 6400	3200
S 11	C 75	200	< 100
C 75	S 11	< 100	< 100
		S 6, 14, 24	C 77
S 6, 14, 24		6400	800
C 77		3200	1600
S 6, 14, 24	C 77	200	< 100
C 77	S 6, 14, 24	< 100	< 100

Table 7 (continued).

O-antiserums	Absorbed by	Titres of O-antigen suspensions	
		S 1, 6, 14, 25	C 77
S 1, 6, 14, 25		800	200
C 77		3200	1600
S 1, 6, 14, 25	C 77	200	< 100
C 77	S 1, 6, 14, 25	< 100	< 100
		S 17	C 85
S 17		3200	400
C 85		1600	1600
S 17	C 85	1600	< 100
C 85	S 17	< 100	< 100
		S 43	C 86
S 43		1600	100
C 86		1600	1600
S 43	C 86	800	< 100
C 86	S 43	< 100	800
		S 1, 6, 14, 25	C 106
S 1, 6, 14, 25		800	800
C 106		1600	1600
S 1, 6, 14, 25	C 106	200	< 100
C 106	S 1, 6, 14, 25	< 100	400
		S 35	C 111
S 35		800	400
C 111		1600	3200
S 35	C 111	200	< 100
C 111	S 35	< 100	1600
		S 47ac	C 118
S 47ac		1600	400
C 118		3200	1600
S 47ac	C 118	200	< 100
C 118	S 47ac	< 100	200
		S 13, 23	C 127
S 13, 23		3200	800
C 127		1600	1600
S 13, 23	C 127	800	< 100
C 127	S 13, 23	< 100	800
		S 39	C OX 9
S 39		800	100
C OX 9		3200	> 6400
S 39	C OX 9	800	< 100
C OX 9	S 39	< 100	1600

strong relationship between nearly all *Arizona* O-antigens and one or another of the *Salmonella* O-antigens, but there are only a few strong O-relationships between these two groups and the *Escherichia* group and there is almost no relationship between them and the *Shigella* group.

The results of the *Arizona-Salmonella* O-relationships are almost identical with these of Edwards and collaborators, but the inter O-group relationships of the *Escherichia* group are in many cases

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TABLE 8.

O-antigen relationships between the groups *Arizona* and *Escherichia*.

O-antiserums	Absorbed by	Titres of O-antigen suspensions	
		C 1	A 15
C 1		6400	800
A 15		800	800
C 1	A 15	3200	< 100
A 15	C 1	< 100	400
		C 2	A 8
C 2		1600	200
A 8		400	1600
C 2	A 8	800	< 100
A 8	C 2	< 100	400
		C 12	A 1, 33
C 12		3200	1600
A 1, 33		400	1600
C 12	A 1, 33	800	< 100
A 1, 33	C 12	< 100	800
		C 21	A 16
C 21		1600	1600
A 16		100	400
C 21	A 16	400	< 100
A 16	C 21	< 100	100
		C 23	A 1, 2
C 23		3200	> 6400
A 1, 2		200	3200
C 23	A 1, 2	800	< 100
A 1, 2	C 23	< 100	200
		C 35	A 6
C 35		3200	> 6400
A 6		400	1600
C 35	A 6	400	< 100
A 6	C 35	< 100	< 100
		C 50	A 8
C 50		6400	> 6400
A 8		400	1600
C 50	A 8	1600	< 100
A 8	C 50	< 100	200
		C 55	A 9ab
C 55		6400	800
A 9ab		1600	3200
C 55	A 9ab	< 100	< 100
A 9ab	C 55	< 100	1600
		C 55	A 9ac
C 55		6400	> 6400
A 9ac		800	3200
C 55	A 9ac	100	< 100
A 9ac	C 55	< 100	< 100
		C 73	A 32
C 73		6400	1600
A 32		400	800
C 73	A 32	800	< 100
A 32	C 73	< 100	100

Table 8 (continued).

O antiserums	Absorbed by	Titres of O-antigen suspensions	
		C 74	A 8
C 74		6400	1600
A 8		400	1600
C 74	A 8	1600	< 100
A 8	C 74	< 100	800
		C 75	A 17
C 75		3200	3200
A 17		800	1600
C 75	A 17	200	< 100
A 17	C 75	< 100	< 100
		C 77	A 7ac
C 77		1600	3200
A 7ac		200	400
C 77	A 7ac	200	< 100
A 7ac	C 77	< 100	< 100
		C 78	A 30
C 78		>6400	3200
A 30		800	1600
C 78	A 30	800	< 100
A 30	C 78	< 100	800
		C 86	A 21
C 86		1600	400
A 21		200	400
C 86	A 21	400	< 100
A 21	C 86	< 100	< 100
		C 111	A 20
C 111		3200	400
A 20		200	400
C 111	A 20	100	< 100
A 20	C 111	< 100	< 100
		C 118	A 28
C 118		1600	3200
A 28		400	800
C 111	A 28	100	< 100
A 28	C 111	< 100	< 100
		C 123	A 1, 33
C 123		1600	>6400
A 1, 33		400	1600
C 123	A 1, 33	100	< 100
A 1, 33	C 123	< 100	< 100
		C 127	A 18
C 127		1600	800
A 18		100	800
C 127	A 18	800	< 100
A 18	C 127	< 100	400
		C OX 13	A 11
C OX 13		3200	100
A 11		400	1600
C OX 13	A 11	200	< 100
A 11	C OX 13	< 100	800

TABLE 9.

O-antigen relationships between the groups *Salmonella* and *Shigella*.

O anti-serums	Absorbed by	Titres of O-antigen suspensions	
		Sh *) A 1	S 42
Sh A 1		> 6400	400
S 42		200	3200
Sh A 1	S 42	1600	< 100
S 42	Sh A 1	< 100	800
		Sh C 3	S 17
Sh C 3		> 6400	400
S 17		200	3200
Sh C 3	S 17	1600	< 100
S 17	Sh C 3	< 100	1600

*) Sh = *Shigella*

TABLE 10.

O-antigen relationships between the groups *Arizona* and *Shigella*.

O antisera	Absorbed by	Titres of O-antigen suspensions	
		Sh A 7	A 14
Sh A 7		> 6400	1600
A 14		1600	3200
Sh A 7	A 14	3200	< 100
A 14	Sh A 7	< 100	800
		Sh B 2a	A 6
Sh B 2a		3200	200
A 6		200	1600
Sh B 2a	A 6	800	< 100
A 6	Sh B 2a	< 100	800

TABLE 11.

Identical O-antigens of the *Salmonella*, *Arizona* and *Escherichia* groups.

O-antigens		
Salmonella	*Arizona*	*Escherichia*
51	1, 2	
44	1, 3	
18	7ab	
50	9ac	55
41	13	
42	15	
38	16	
11	17	
35	20	111
21	22	
47ab	23	
16	25	
	28	118
52	31	
6, 14, 24	32	
	1, 33	123

different from the findings of Ewing and associates. Not only are the *E. coli* antigens highly agglutinable with anti-serums of the other groups, but the exact strains and serums used must be taken into account. Another set of *E. coli* strains and/or serums may give quite different results. The ease of agglutination with anti-serums of different groups is seen not only in the *Escherichia* group, but also in the *Shigella* group. These antigens are agglutinated easily by anti-serums of the *Salmonella* and *Arizona* groups, but *Shigella* anti-serums agglutinate only a few antigens of the latter groups.

The results of these investigations may be important for the daily routine of laboratories working with *Enterobacteriaceae* in the following way. They show clearly, that it is impossible to prepare a special diagnostic polyvalent serum, which is absolutely group-specific. There are no antigenic frontiers within the Family *Enterobacteriaceae* and it is therefore useless to prepare, for example, so-called "specific polyvalent *Salmonella* serums", as done in recent years by commercial producers.

Both negative and positive reactions with a polyvalent group-serum give only an indication for a diagnosis. Group and type determination must be done by both serological and biochemical methods. Only the results of both the methods will give the accurate diagnosis, which is important to the epidemiologist.

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Summary.

An account is given of O-relationships between the groups

Salmonella (O-antigens 1—52), *Arizona* (O-antigens 1, 2—1, 33) *Escherichia* (O-antigens 1—142 and OX 1—OX 13) and *Shigella* (O-antigens A1 — A10, B 1a — B6, C1 — C15 and D). Through cross agglutination studies and absorption tests the instances of O identity in antigens of the different groups were determined.

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"L" FORMS OF STAPHYLOCOCCI; THEIR REVERSIBILITY; CHANGES IN THE SENSITIVITY PATTERN AFTER SEVERAL INTERMEDIARY PASSAGES IN THE "L" PHASE

by

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with the technical assistance of H. A. DE HAAS-POPPINGA

(Received March 12, 1959).

Dienes and Sharp (1956) were the first to describe "L" forms of Staphylococci. Using their medium we could confirm and even extend their results. We regained the bacterial form even after more than twenty passages in the "L" phase.

Taking the resistance to one or to two antibiotics as a marker, we were able to identify the isolated Staphylococci. In nearly all the series the phage patterns of the isolated strains could be determined. For this typing we gratefully acknowledge our indebtedness to Professor Ruys (University of Amsterdam).

Methods.

Recently isolated strains as well as strains from the laboratory collection gave equally good results. All strains mentioned below were sensitive to penicillin. Strains No. 6117 and No. 6105 were made resistant *in vitro*, respectively to streptomycin and tyrothricin; strains No. 5124 and No. 1724, when isolated, were found to be already resistant to chloramphenicol, No. 3525 and No. 163 to tetracycline and No. 3536 to tetracycline and erythromycin. Strain 6114 was sensitive to all antibiotics.

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In preliminary experiments we used DIENES' medium containing 3.7 per cent brain-heart infusion, 1.3 per cent agar, 3 per cent salt, 10 per cent inactivated horse serum and 100 U/ml of penicillin. In later experiments the serum was replaced by 20 per cent ascitic fluid, with slightly better results. Apart from occasional unexplained failures, the results were usually satisfactory. The media were inoculated by spreading several drops of a dense 24 hour culture on the surface. Aerobic and anaerobic incubations proved to be equally effective. In order to prevent exsiccation, the cultures were sealed.

For serial transfer in the "L" phase, the agar block technique was used. To recover the bacterial form, a high concentration of penicillinase [1]) was dripped onto the surface of the medium containing fully developed "L" colonies, or the agar block, carrying the "L" colonies, was applied to a fresh penicillin-free DIENES' medium. In the bacterial phase, subcultures were made by replicating 50 separate colonies, using the blotting paper discs as previously described (SCHÖNFELD, 1955).

The following properties of the recovered Staphylococci were examined:

1. phagepattern;
2. sensitivity to penicillin and to the marker antibiotic(s);
3. coagulase production;
4. pigment formation;
5. fermentation of mannitol;
6. haemolysin.

In order to minimize the influence of phagal variation the specimens from each series were typed at the same time. The technique of phagetyping and the interpretation of the results will be described elsewhere (RUYS and BORST, 1959).

Parallel sensitivity tests of the recovered strains were done by the diffusion method (antibiotics incorporated in paper discs) and by the dilution method (serial dilution of antibiotics in blood agar plates). The results of these two methods were identical in all cases. Coagulase production was tested with bovine plasma (BOSGRA, 1949).

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[1]) Kindly supplied by the "Koninklijke Nederlandsche Gist- en Spiritusfabriek", Delft.

RESULTS.

Twenty strains sensitive to penicillin were examined; each could be transformed at least once into the "L" form and bacteria were recovered from five or more "L" passages. Two strains were stabilized in the "L" form in the 9th and 11th passage respectively.

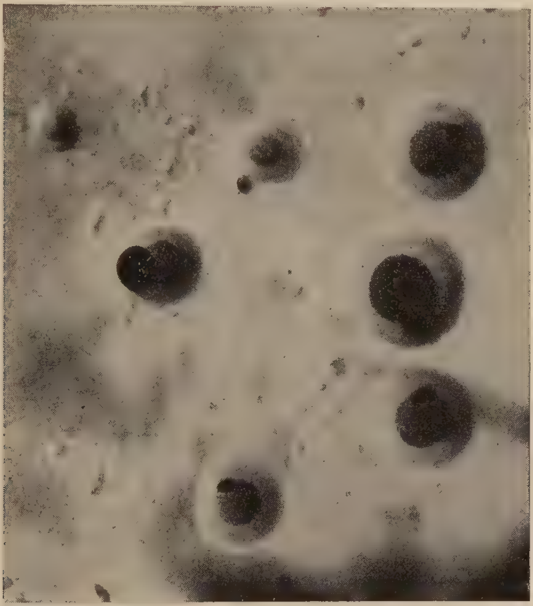

Fig. 1. Small staphylococcal colonies developing from "L" colonies (5th passage) 26 hours after addition of penicillinase.

The "L" colonies usually appeared after 24 to 48 hours' incubation. 24 hours after the addition of penicillinase, bacterial colonies were visible (fig. 1). An illustration of the results of the phagetyping is given in table 1. Apart from the 7th and 24th passages the pattern was very well maintained. According to Professor RUYS' experience changes in the number of active phages as well in the degree of their activity - as observed in strain 6114 and in some other strains - may have been the result of bacterial variation. We never found new types.

The sensitivity pattern of the isolated strains are represented in figure 2. The resistance to the marker antibiotic, chloramphenicol, was obviously lost after two or three passages in the "L" phase.

TABLE 1.

Strain No. 6114. Phage pattern of 25 passages in the "L" form.

	0	/52, 29/79, 52A, 80.
L	1	/52/29, 52A, 79, 80.
L	2	/52, 29, 52A, 79, 80/47, 53, 6, Nobel, 42E.
L	3	/52, 79, 52A, 29, 80/53, 47, 6, Nobel, 44B, 7, 73.
L	4	/52, 29, 52A, 79, 80/6, 47, 53, Nobel, 73.
L	5	/52, 29,/52A, 79, 80, 47, 6, 53, Nobel.
L	6	52/80, 52A,/29, 79, 47, 53, 6, Nobel, 42E.
L	7	untypable.
L	8	/52/29, 52A, 79, 80, 47, 53, 6, Nobel.
L	9	/52, 52A, 79, 80, 29/53, 47, Nobel, 6, 42E.
L	10	29, 52, 80/52A, 79/6, 47, 53, Nobel, 44B, 42E, 44.
L	11	/52, 52A, 29, 80/79, 47, 53.
L	12	/52, 29, 79, 52A/80, 6, 47, 53, Nobel, 42E.
L	13	52/29, 79, 80, 52A/44, 55, 3C, 71, 6, 54.
L	14	/52, 52A, 79, 80, 29/47, 53, 6, Nobel, 7.
L	15	/52, 80, 52A, 29/79, 6, 47, 53.
L	16	/52, 80/79, 52A, 29, 6, 47, 53.
L	17	/52, 29, 79, 52A, 80/47, 6, 53, Nobel, 42E.
L	18	/52/80, 52A, 79, 29.
L	19	29, 52, 80, 52A/79/53, 6, 47, 44B, Nobel, 42E.
L	20	/52, 80, 52A, 79, 29/47, 6, 53, 42E, 44, Nobel.
L	21	/52/80, 52A, 79, 29, 53, 47, 6, Nobel.
L	22	53/6, 47, 7, 54/77, 52, 52A, 79.
L	23	/52, 80/52A, 79, 53, 6, 47.
L	24	lost owing to contamination.
L	25	52/44, 29, 70/80, 73, 52A, 79, 42D.

After three to eleven passages strains, originally resistant to tetracycline and erythromycin, were found to be sensitive to these antibiotics. These strains were isolated from patients. Strains which were made resistant *in vitro* to streptomycin or tyrothricin remained consistently insensitive throughout twenty passages. No change in the sensitivity to penicillin was observed in any of these experiments. When strain 5124, originally resistant to chloramphenicol, and 3525, resistant to tetracycline, were transformed alternatively to the "L" phase and reversed to the coccal phase no loss of resistance to the marker antibiotic was observed throughout 15 consecutive passages. In the same experiment strain 3536 lost the resistance to tetracyline after the 14th passage, but the strain was still insensitive to erythromycin after 17 passages. From the original strain 5124 nearly 20 per cent of the organims proved to be sensitive

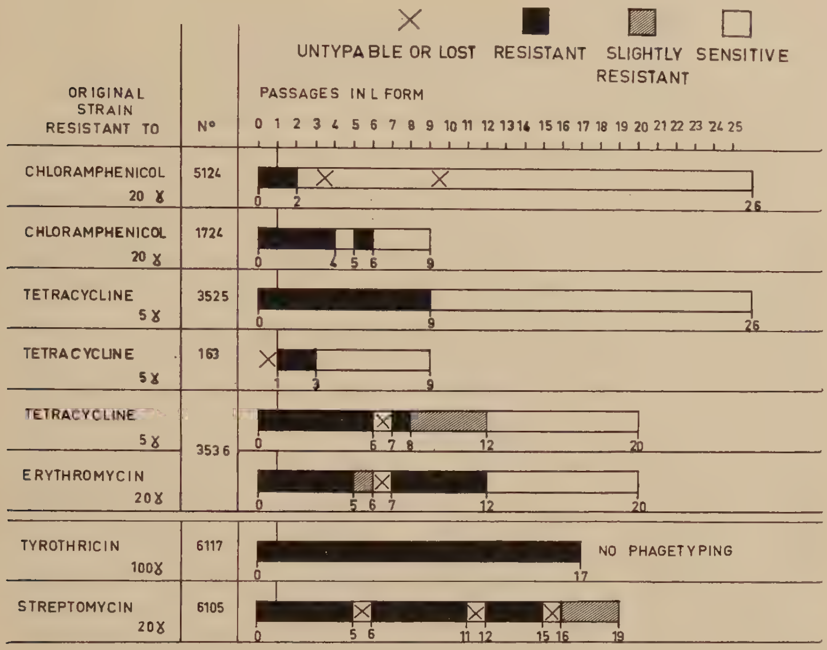

Fig. 2. Sensitivity pattern of strains recovered from the "L" phase.

to chloramphenicol. This phenomenon was described earlier by CHABBERT and DEBRUGE (1956). Of the resistant substrains, subsequently 2 per cent were found to be sensitive. From 50 fully developed colonies originating from individual bacteria, 38 colonies maintained their resistance throughout 15 passages in the bacterial form. From strains 3525 and 3536 no sensitive substrains were ever isolated, when 50 colonies grown from individual bacteria, were subcultured for 15 passages.

DISCUSSION.

Although the reversibility of the "L" phase to the bacterial phase has been described nearly as often as the transformation into the "L" form itself, the properties of the bacteria, recovered after serial "L" passages, have received only scanty attention. SHARP, DIENES and HIJMANS (1957) found the "L" form of group A Streptococci to lack the group specific polysaccharide, but they noticed that bacteria reversed from the first "L" passage recovered the ability to produce this carbohydrate. Aktan and Fisek (1957), however, mention important biochemical and serological changes, found in *Enterobacteriaceae* after one "L" passage or two alternate transformations. Lederberg and St. Clair (1958), who made numerous serial "L" passages using the *E. coli* K 12 and substrains, state that the reversed cultures were indistinguishable from the original in their growth characteristics and that the wall defect imposed by penicillin had no effect on the genetic continuity. In our experiments the change from resistance to sensitivity was repeatedly observed with chloramphenicol, tetracycline and erythromycin in five different strains. This loss of resistance is not likely to be the result of bacterial mutation, for it was observed only once in three series of 15 consecutive alternate transformations into the "L" form and back to the coccal form. Sensitive substrains never appeared when tetracycline or erythromycin resistant strains were subcultured exclusively in the coccal form. Of strains partially resistant to chloramphenicol the properties of sensitive substrains gradually increased. After 15 passages 2/3 of the colonies were still resistant, however.

From the experiments it may be concluded that the transformation of Staphylococci into "L" forms is reversible. Even after twenty five passages the coccal form was repeatedly regained. As in the case of many other bacteria an "L" cycle of Staphylococci is found to exist. The loss of resistance to some marker antibiotics (chloramphenicol, tetracycline and erythromycin) was found to appear after a number of passages in the "L" form. This property appeared to be the only significant variation form the original strain.

Summary.

Strains of Staphylococci originally resistant to chloramphenicol, tetracycline and erythromycin, or made resistant *in vitro* to tyrothricin and streptomycin, were transformed into the "L" form and subcultured in this phase for more than twenty passages. The "L" colonies of the entire series could be reversed to bacterial forms. From all the isolated staphylococci strains the phage pattern was found to be maintained; slight changes in the number of active phages and the degree of activity are deemed to be insignificant. New types were never found. The strains made resistant to tyrothricin and streptomycin remained insensitive, those, however, that

were originally resistant to chloramphenicol, tetracycline and erythromycin, became sensitive after a number of passages in the "L" phase. This loss of resistance - the only significant change from the original strains - is thought not to be due to mutation.

Résumé.

Des souches de Staphylocoques sensibles à la pénicilline, mais naturellement résistantes au chloramphénicol, à la tétracycline et à l'érythromycine, aussi bien que des souches rendues insensibles à la tyrothricine ou à la streptomycine, étaient transformées en phase "L". Dans cette phase plus de vingt subcultures étaient faites; les colonies "L" de chaque passage pouvaient être reversées en phase bacillaire. Toutes les souches récupérées avaient conservé le type bactériophagique des souches d'origine; l'augmentation du nombre des phages actifs et le changement du degré de leur activité ne peuvent être considérés signifiants. Nous n'avons jamais trouvé de nouveaux types.

Les souches résistantes à la streptomycine et à la tyrothricine restaient insensibles; celles au contraire, qui étaient naturellement résistantes a la tétracycline, à l'érythromycine ou au chloramphénicol devenaient sensibles après peu de subcultures en phase "L". Cette perte de résistance est considérée être d'origine non-mutationelle.

L'existence d'un cycle "L" des Staphylocoques s'est averée ainsi.

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THE OXIDATION OF STRAIGHT AND BRANCHED ALKANES BY *PSEUDOMONAS* STRAINS

by

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(Received May 31, 1959).

INTRODUCTION.

Microbiological oxidation of alkanes has been studied occasionally since the beginning of this century. A correlation between oxidizability and alkane structure could, however, not be established mainly owing to the fact that suitable series of samples of pure alkanes were not available (BEERSTECHER 1954). As a result the information obtained so far is scattered and often contradictory.

Iso-octane (2.2.4-trimethylpentane) for example, was shown to be oxidized by marine and soil bacteria, (ZOBELL 1943, and STRAWINSKI 1940, respectively) whilst KONOVALTSCHIKOFF and SENEZ (1956) found this hydrocarbon to be quite refractory against attack by *Pseudomonas* strains. Experiments by KERELIN and VOROB'EVA (1957) suggest that straight chains facilitate oxidation, whereas most authors of review articles (ZOBELL 1950, STEEL 1957 and BEERSTECHER 1954) conclude that branched alkanes are degraded more readily by micro-organisms than the *n*-alkanes.

The present work aims at throwing some new light on this question. It is concerned with the investigation of the oxidizability of 53 pure alkanes by two *Pseudomonas* strains.

EXPERIMENTAL.

Organism.

The isolation of a *Pseudomonas* strain (no. 473) from IJ-harbour (Amsterdam) water on a kerosine medium and the identification of the organism as *Ps. aeruginosa* have been described before (THIJSSE and VAN DER LINDEN 1958). Recently a new *Pseudomonas* strain (no. 475) was isolated from the same source using hexadecane as the sole source of carbon in the enrichment culture. This strain was not further identified. It was used in the determination of the oxidizability of the hexadecane isomers summarized in Table 2.

Alkanes.

The alkanes mentioned in Table 1 were obtained from the American Petroleum Institute or from the Phillips Petroleum Cy. Their purities are claimed to be 99.2 mol % or better.

The hexadecane isomers from Table 2 where synthesized by WIBAUT and BRAND (1959). The purities of these samples were calculated from the melting curves according to a modified method of the National Bureau of Standards using a eutectic system (MAIR *et al.* 1941 and MALOTAUX 1933 and 1934). They were found to be 99.2% or better (BRAND 1956).

Methods.

Growth of the micro-organism in the presence of an alkane as the sole source of carbon was taken as a criterion of the oxidizability of that particular alkane. No doubtful cases were encountered in the examination for visible growth after a 3 week incubation period at 30°C.

Liquid media were used in most cases; some alkanes were also tested using solid media.

Liquid medium test: Sterile Erlenmeyer flasks of 200 ml provided with a ground glass stopper were filled with 50 ml sterile mineral medium (KH_2PO_4 4.5 g; Na_2HPO_4 4.7 g; KNO_3 1 g; $FeSO_4$ trace; Tap water 1000 ml; pH 6.8).

One or two drops of the hydrocarbon were added and the flasks were incubated at 30°C. after inoculation. Turbidity observed after 3 weeks was compared with an inoculated but hydrocarbon-free blank.

Solid medium test: Petri dishes with mineral agar (1.8% agar) were inoculated. A strip of filter paper provided with a drop of the alkane was placed in the lid of the dishes. Incubation at 30°C. was performed in sealed tins. Growth was compared with that obtained in inoculated but hydrocarbon-free dishes.

Low-boiling hydrocarbons were introduced as a mixture with a high-melting paraffin to reduce the vapour pressure. In this modification the blanks were prepared with pure paraffin in the lids of the inoculated Petri dishes.

RESULTS.

The results obtained with *Pseudomonas* 473 (*Ps. aeruginosa*) are presented in Table 1. It can be seen from this table that all straight alkanes from C_5 to C_{16} are oxidized, whereas the compounds containing one methyl substituent are attacked only when their molecules contain a sufficiently long unbranched chain (at least 3 or 4 carbon atoms). Comparison of the oxidizability of 3-methylhexane (p. 99.6 mol %), 3-methylheptane (p. 99.2 mol %) and 4-methylheptane (p. 99.8 mol %) shows that there is no simple relation between oxidizability and the minimum length of the unbranched chain. Substitution by larger alkyl groups or by more than one methyl group always prevented the oxidation in a 3 week period.

TABLE 1.

Oxidation of straight and branched alkanes by
Pseudomonas 473 after 3 weeks incubation at 30° C.

n-pentane	+	*n*-octane	+
		2-methylheptane	+
n-hexane	+	3-methylheptane	+
2-methylpentane	—	4-methylheptane	+
3-methylpentane	—	3-ethylhexane	—
2.2-dimethylbutane	—	2.2-dimethylhexane	—
2.3-dimethylbutane	—	2.3-dimethylhexane	—
		2.4-dimethylhexane	—
n-heptane	+	2.5-dimethylhexane	—
2-methylhexane	+	3.3-dimethylhexane	—
3-methylhexane	—	3.4-dimethylhexane	—
3-ethylpentane	—	2-methyl-3-ethylpentane	—
2.2-dimethylpentane	—	3-methyl-3-ethylpentane	—
2.3-dimethylpentane	—	2.2.3-trimethylpentane	—
2.4-dimethylpentane	—	2.2.4-trimethylpentane	—
3.3-dimethylpentane	—	2.3.3-trimethylpentane	—
2.2.3-trimethylbutane	—	2.3.4-trimethylpentane	—
		2.2.3.3-tetramethylbutane	—
		n-nonane through *n*-hexadecane	+

The same rules apply to the results of Table 2 obtained with *Pseudomonas* 475. There is, however, one exception here. Among the compounds tested having a side chain of two or more C atoms there is one, 3-ethyltetradecane (p. 99.8 mol. %), which is oxidized within the 3 week period.

TABLE 2.

Oxidation of C_{16} alkanes by *Pseudomonas* 475 after 3 weeks incubation at 30° C.

n-hexadecane	+	3-ethyltetradecane	+
2-methylpentadecane	+	4-propyltridecane	—
3-methylpentadecane	+	7-propyltridecane	—
4-methylpentadecane	+		
5-methylpentadecane	+	5-butyldodecane	—
6-methylpentadecane	+		
7-methylpentadecane	+	6-pentylundecane	—
8-methylpentadecane	+		
		4.7-dipropyldecane	—

Considerable efforts were made to isolate a micro-organism oxidizing *iso*-octane. The use of prolonged adaptation periods and of various source materials did not meet with success, however. In addition it was tried to adapt *Pseudomonas* 473 to *iso*-octane. Growth of the micro-organism in these experiments was sustained during the incubation period by adding 0.1 mol. % *n*-octane to the *iso*-octane. Subculturing in this way for several weeks did not alter the specificity of the micro-organism towards *n*-octane and *iso*-octane. Growth as measured by turbidity remained proportional to the amount of *n*-octane present in *iso*-octane samples in the 0.01—0.1 mol. % *n*-octane range (10 days incubation at 30°C.; 3 ml hydrocarbon mixture; 50 ml mineral medium). In fact a microbiological assay may be based on the phenomenon.

Our results show that branched alkanes in general (and *iso*-octane in particular) are quite refractory to oxidation by *Pseudomonas*. This stands in contrast to what is mentioned in most review-papers on alkane oxidation but agrees with results of investigations on the biological hardness of detergents (alkylbenzene sulfonates etc.), which demonstrate that branching increases biological hardness (SAWYER 1958, BOGAN and SAWYER 1955 and HAMMARTON 1955).

Summary.

A number of straight and branched alkanes were tested with respect to their oxidizability by *Pseudomonas*.

n-Alkanes are easily oxidized, but the branched compounds must possess a sufficiently long unbranched chain in order to be attacked.

With one exception no oxidizable compounds were encountered among those containing more than one methyl substituent or one or more higher alkyl substituents.

Acknowledgment.

We are greatly indebted to the late Professor KLUYVER (Delft) for his interest and valuable advice.

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PICHIA ROBERTSII NOV. SPEC.
A NEW HAPLOID HOMOTHALLIC YEAST

by

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(Received May 4, 1959).

The larval feed of the South African bumble bee, *Xylocopa caffra* contains yeasts in considerable numbers. From a series of samples of these pollen-pabulums collected in Pretoria, the following yeast species were identified: *Torulopsis magnoliae*, *Torulopsis famata*, *Candida reukaufii*, *Saccharomyces cerevisiae* together with a hitherto undescribed new species. As the latter is of taxonomic interest, a description is given which is based on the standard procedure of Lodder and Kreger-van Rij (1952) and Wickerham (1951, 1957a).

Description.

Growth in malt extract: After 3 days at 25°C., the cells are round to short oval (2.3 — 7.5) × (3.1 — 8.8) μ, single, in pairs or clusters. A sediment and thin dull creeping pellicle are formed. After 1 month at 17°C., only a sediment is present.

Growth on malt agar: After 3 days at 25°C., the cells have the same shape and size as in malt extract.

After 1 month at 17°C., the streak culture is raised, yellowish brown with dark brown sectors, with little warts which are either white or dark brown. The culture is somewhat shiny but not mucoid. The margin is undulating.

Slide cultures: No mycelium or pseudomycelium is formed.

Sporulation: The vegetative cells are haploid. A heterogamous mother-daughter cell conjugation usually precedes ascus formation. Very occasionally independent cells conjugate prior to ascus formation. Cells with protuberances are formed. The ascospores are oblate ellipsoidal, smooth and yellowish brown in colour, and contain oil droplets; 1—4 are formed per ascus.

Fermentation:

Glucose	+	Maltose	+
Galactose [1]	—	Lactose	—
Saccharose	+	Raffinose	1/3
Trehalose	+	Melizitose	—

Carbon assimilation: Glucose, galactose, sorbose, maltose, saccharose, cellobiose, trehalose, raffinose, melizitose, D-xylose, L-arabinose, D-ribose (latent), L-rhamnose, ethanol, glycerol, erythritol, adonitol, mannitol, sorbitol, α-methyl-D-glucoside, salicin, potassium-D-gluconate, DL-lactic acid (latent), succinic acid, and citric acid are assimilated. Melibiose, lactose, D-arabinose, dulcitol and m-inositol are not assimilated.

Assimilation of nitrate and nitrite: Absent.

Splitting of arbutin: Positive.

Growth in the absence of an extraneous vitamin source: Absent.

DISCUSSION.

With the present status of the taxonomy of the nitrate-negative, haploid homothallic yeasts which show a more or less predominant heterogamous mother-daughter cell conjugation, no entirely satisfactory generic classification of the yeast in question can be given.

WICKERHAM (1951, 1957b) and WICKERHAM and BURTON (1954, 1956a, 1956b) in their studies on the genus *Hansenula* and the proposed genus *Dekkeromyces* showed that the only reasonable basis for the definition and demarcation of a natural genus is the study of the species of the genus along evolutionary phylogenetic lines. From their experience with these two genera they formulated the following evolutionary trends which exists in such phylogenetic lines:

(*a*) The fundamental trend of a progressive change from the haploid state in the most primitive species to the diploid state in the most recently evolved species. It was further indicated that the most primitive sexuality involved the conjugation of a large cell with its own bud.

(*b*) A change from dependence on to independence of an extraneous vitamin source.

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[1] Recently a second strain of *Pichia robertsii* has been isolated which ferments galactose vigorously.

(*c*) An increase in fermentative ability.

(*d*) A progressive increase in the ability to produce esters.

(*e*) Progressive changes in morphological properties. The most primitive species form mucoid colonies which gradually give way to a mat or rugose colony-form in the more recently evolved species. The ability to produce mycelium or pseudomycelium, as well as the formation of a pellicle, generally increases as the species become more diploid.

If these evolutionary criteria are applied to our yeast, the following becomes apparent: The species exists in the haploid phase and shows a characteristically primitive sexuality in that a mother-daughter cell conjugation prevails. This rates the species as rather primitive. This is further supported by the fact that this species is dependent on an extraneous vitamin source. On the other hand, the species has a strongly fermentative dissimilation, fermenting at least five sugars. This suggests that it has been more recently evolved. This is supported by the fact that it produces a pellicle and forms rugose rather than mucoid colonies on solid media.

The above suggests that the evolutionary development of the sexual, biochemical and morphological characteristics in a phylogenetic line need not be synchronized or at least need not proceed at the same rate as was observed in the genera *Hansenula* and *Dekkeromyces*. This implies the possibility of the existence of sexually primitive phylogenetic lines in which a unilateral development of the biochemical and morphological characteristics has taken place.

To define a phylogenetic line, the fundamental generic characteristics must be well expressed. In the genus *Hansenula* these were the assimilation of nitrate together with the absence of interfertility amongst the different species. In the genus *Dekkeromyces*, they were interfertility and the more or less rapid release of the mature ascospores.

In the case of the yeast described here, the fundamental generic characteristics are at present not as obvious. Interfertility or its absence as a criterion is extremely difficult to verify in the case of haploid homothallic yeasts where mother-daughter cell conjugation predominates. Other characteristics which may be of use in its classification are:

(*a*) The rather unusual oblate-ellipsoidal ascospore shape. The value of this characteristic is difficult to assess since it has not been encountered before and also since a fairly wide variation in ascospore shape may exist within a genus as has been shown in the genera *Hansenula* and *Dekkeromyces*.

(*b*) The maximum spore number of four.

(*c*) The rather polyphage carbon assimilation.

(*d*) The difference between the degree of development between the sexual and biochemical evolutionary characteristics. To the author this appears to be the most useful characteristic for the generic classification of the species.

At present there are three groups of yeasts which can be taken into consideration and which show the same unequal evolution of sexual and biochemical characteristics. These are:

Group 1 - This includes yeasts which were previously classified as *Torulospora* species but are now classified in the genus *Saccharomyces*, *viz.*, *Sacch. rosei* (Guilliermond) Lodder et Kreger-van Rij 1913, *Sacch. fermentati* (Saito) Lodder et Kreger-van Rij 1923, *Sacch. microellipsoides* Osterwalder 1924, *Sacch. florentinus* (Castelli) Lodder et Kreger-van Rij 1938, *Sacch. pretoriensis* van der Walt et Tscheuschner 1956 as well as *Debaryomyces globosus* Klöcker 1909. This group has a marked fermentative dissimilation. It includes species like *Sacch. rosei* which ferments three sugars and which forms no pseudomycelium as well as species like *Sacch. florentinus* which ferments at least six sugars and which forms a well-developed pseudomycelium. None of these species are polyphage in their carbon assimilation. None form more than four spores per ascus. Nor do they form pellicles. Hence it is not possible to include the yeast from the pollen-pabulum in this group.

Group 2 - This consists of species which are at present classified in the genera *Debaryomyces* (*in sensu* Lodder et Kreger-van Rij) and *Pichia* (*in sensu* Phaff), *viz.*, *Debaryomyces hansenii* (Zopf) Lodder et Kreger-van Rij 1889, *Debaryomyces kloeckerii* Guill. et Péju 1919, *Debaryomyces subglobosus* (Zach) Lodder et Kreger-van Rij 1934, *Debaryomyces nicotianae* Giovannozzi 1939 and *Pichia polymorpha* Klöcker 1912. This group is polyphage in its carbon assimilation. The metabolism is predominantly oxidative but there appears to be a gradient in the fermentative abilities. These may be absent or weak in some species but well developed in others, *e.g.*, *Pichia polymorpha*, which ferments at least five sugars. Likewise there is a transition in the formation of a pellicle and pseudomycelium. These are absent in species like *D. subglobosus.* Mother-daughter cell conjugation predominates. No more than two spores per ascus are formed. The latter characteristic excludes the species from the pollen-pabulum from this group. WICKERHAM (1957a) has indicated that some members of this group may assimilate nitrite but not nitrate.

Group 3 - This group includes various *Pichia* species which were classified in the genus *Debaryomyces* (*in sensu* Lodder et Kreger-van Rij) and which PHAFF (1956) has suggested should be transferred to genus *Pichia, viz., Pichia vini* (Zimmerman) Phaff 1938, *Pichia marama* (di Menna) Phaff 1954, *Debaryomyces tolentanus* Socias, Ramirez et Peléaz 1954, *Pichia vanriji* van der Walt et Tscheuschner 1956, *Pichia farinosa* (Lindner) Hansen 1894 and *Pichia pseudopolymorpha* Boidin et Ramirez 1953. This group is more or less polyphage in its carbon assimilation. The metabolism ranges from strictly oxidative in species like *Pichia marama* to species with a well-developed fermentative ability like *Pichia farinosa* which ferments two sugars and *Pichia pseudopolymorpha* which ferments at least five sugars. There is also a gradient in the formation of a pellicle and pseudomycelium. These are absent in species like *Pichia marama* but well developed in *Pichia farinosa* and *Pichia pseudopolymorpha.* Mother-daughter cell conjugation predominates. Not more than four spores per ascus are formed.

In the author's opinion, the yeast from the pollen is best included in this third group.

It is difficult at the moment to assess the precise status of these three groups and particularly the relationship between groups two and three. PHAFF's amendment of the diagnosis of the genus *Pichia* has been of great practical value for the classification of the haploid homothallic yeasts in which mother-daughter cell conjugation predominates. The present demarcation of this genus is, however, unavoidably vague. This has led to the genus becoming rather heterogeneous. In its present form it probably incorporates more than one phylogenetic line. It is difficult, for example, to conceive a single phylogenetic line which includes species in which mother-daughter cell conjugation seldom occurs and which are not polyphage in their carbon assimilation, *e.g., Pichia membranaefaciens,* as well as species in which mother-daughter cell conjugation predominates and which are polyphage and strongly fermentative, *e.g.*, *Pichia pseudopolymorpha*. The species of the genus *Petasospora* (Boidin and Abadie, 1954) which Phaff (1956) suggested should be included in the genus *Pichia* probably constitute another phylogenetic line which does not include the type species of the genus *Pichia*, *viz.*, *Pichia membranaefaciens*.

It therefore seems inevitable that the genus *Pichia* must eventually be redefined. This makes a choice of a generic name for the yeast described in this communication awkward. For practical purposes the author prefers to place it provisionally in the genus *Pichia* (*in sensu* Phaff) until such time as its true generic position can be established.

For the species the name *Pichia robertsii* is proposed in honour of Dr Catherine Roberts for her meritorious contributions in yeast genetics.

Cultures of *Pichia robertsii* have been deposited in the yeast collection of the Centraal Bureau voor Schimmelcultures in Delft.

Pichia robertsii nov. spec.

In extracto malti cellulae rotundae aut subovoidiae (2.3—7.5) × (3.1—8.8) μ, singulae, binae aut in racemis parvis. Post dies 3 pellicula tenuis, non-nitida, sursum repens formantur. Sedimentum.

In agar malti formae et dimensiones cellularum eaedem sunt que in extracti malti. Cultura (post unum mensem 17°C.) flavifusca cum fuscis sectoribus, parum acclivis, cum verrucis parvis, aliis albis et aliis fuscis, parum nitens. Margine parum undulato.

Pseudomycelium aut mycelium nullum. Cellulae vegetativae haploideas. Asci ex conjugatione heterogamica inter cellulas maternas et earum gemmas; perraro ex conjugatione cellularum liberarum. Cellulae quoque cum tuberculis similibus tubis conjugulis. Ascosporae sunt in conformatione globi circa extremas partis axis depressiores ac planiores et globulos olei continent, glabrae, colore fusci pallida; 1—4 in asco.

Glucosum, saccharosum, maltosum, trehalosum et raffinosum (pro tertia parte) fermentatur at non galactosum, melizitosum nec lactosum.

Glucosum, galactosum, sorbosum, maltosum, saccharosum, cellobiosum, trehalosum, raffinosum, melizitosum, D-xylosum, L-arabinosum, D-ribosum, L-rhamnosum, ethanolum, glycerolum, erythritolum, adonitolum, mannitolum, sorbitolum, α-methyl-D-glucosidum, salicinum, kalium D-gluconicum, DL acidum lacticum, acidum succinicum et acidum citricum assimilantur at non melibiosum, lactosum, D-arabinosum, dulcitolum nec inositolum.

Kalium nitricum et natrium nitrosum non assimilantur. Arbutinum finditur.

Necessariae ad crescentiam sunt vitaminae externae. Isolata ex pabulo larvali *Xylocopae caffrae*.

Summary.

A new haploid, homothallic yeast isolated from the larval feed of *Xylocopa caffra* is described.

A study of its properties shows that the evolution of its sexual and biochemical characteristics has not taken place at the same rate as in the genus *Hansenula* and the proposed genus *Dekkeromyces*. This led the author to the conclusion that in the yeast domain phylogenetic lines may exist in which a unilateral evolution of biochemical characteristics has taken place.

At least three groups of yeasts exist where such discrepancies exist between the evolution of their sexual and biochemical characteristics. The inclusion of the new species in these groups is discussed.

The new species has provisionally been classified in the genus *Pichia* (*in sensu* Phaff).

A Latin diagnosis of the species is given.

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ENDOMYCOPSIS WICKERHAMII NOV. SPEC. A NEW HETEROTHALLIC YEAST

by

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(Received May 4, 1959).

WICKERHAM (1957) has demonstrated the strict habitat-specificity which certain *Hansenula* species exhibited for coniferous trees and the insects associated with these. He suggested to the author that it might be of interest therefore to attempt to isolate yeasts which could possibly be associated with the *Cycadales* — an order of primitive African gymnosperms.

During the course of isolations from the larval gut and frass of a member of the *Cossidae* infesting a microsporangiate strobilus of a natural cycad hybrid (*Encephalartos altensteinii* × *Encephalartos lehmanii*) from the Port Elizabeth area, several strains of a new yeast were encountered. As this species is of taxonomic interest, a description is given. This is based on the standard methods of LODDER and KREGER-VAN RIJ (1952) and WICKERHAM (1951).

DESCRIPTION.

Growth in malt extract: After 3 days at 25°C., the cells are short-oval, long-oval to elongate (1.9—6.3) × (3.5—31.0) μ, single, in pairs or in branched chains. Mycelial hyphae are also present (2.5 — 4.8) μ in diameter. A thin creeping pellicle and sediment are formed.

After 1 month at 17°C., a sediment, ring and pellicle are present.

Growth on malt agar: After 3 days at 25°C., the cells are short-oval, long-oval to elongate, (1.8—5.0) × (2.5 — 31.0) μ, single or in pairs or branched chains. A true mycelium, (1.9 —3.8) μ in diameter is present.

After 1 month at 17°C., the culture is brownish-white, raised in the middle with fine structures and somewhat shiny. The margin is filamentous.

Slide cultures: A very well-developed pseudo-mycelium and also true mycelium is formed.

Sporulation: Asci arise either from the direct transformation of diploid vegetative cells or after conjugation of haploid vegetative cells of opposite sexuality. 1—4 Hat-shaped spores are produced per ascus. The asci rupture soon after maturation.

Fermentation:

Glucose	+	Lactose	—
Galactose	—	Raffinose	—
Saccharose	—	Melibiose	—
Maltose	—		

Carbon assimilation: Glucose, saccharose, maltose, cellobiose, trehalose (weak), melizitose, xylose, rhamnose, ethanol, glycerol, adonitol, mannitol, sorbitol, α-methyl-D-glucoside, salicin, potassium D-gluconate, calcium 2-keto-D-gluconate (weak), potassium 5-keto-D-gluconate, pyruvic acid, DL-lactic acid, succinic acid, citric acid are assimilated. Galactose, sorbose, lactose, melibiose, raffinose, inulin, soluble starch, D- and L-arabinose, D-ribose, erythritol, dulcitol, potassium-sodium saccharate and i-inositol are not assimilated.

Assimilation of nitrate and nitrite: Absent.

Splitting of arbutin: Positive.

Growth in osmotic pressure medium: Positive.

Growth in absence of extraneous vitamin source: Absent.

Growth at 37°C.: Positive.

DISCUSSION.

The generic position of the species in question is at present debatable.

WICKERHAM and BURTON (1954b) in their studies of the mating types and perfect forms of *Candida guilliermondii* (Cast.) Langeron et Guerra and *Candida membranaefaciens* Wickerham et Burton first established that these nitrate-negative, heterothallic yeasts, which exist either as bisexual diploids or haploid mating types and which produce hat-shaped ascospores which are soon released after maturation, form a well-defined natural group. The American authors, however, advocated retaining this group in which interfertility is absent, in the genus *Endomycopsis* until such time as a more precise definition and delimination of the new group was possible.

A survey of the properties of the strains from the cycad strobilus clearly indicates their incorporation in this group.

Boidin and Abadie (1954) have, however, erected the new genus *Petasospora* to incorporate a number of sporogenous yeasts, all primarily isolated from plant sources. The genus was defined as: "Cells round, oval or elongate; pseudomycelium present or absent; multipolar budding; spores hat- or saturn-shaped which are easily liberated from the ascus; conjugation may precede ascus formation. In liquid media bottom growth, ring, and after longer time, a mucous pellicle. Fermentation variable and may be absent. Nitrate not assimilated".

Although the French authors paid very little attention to the sexuality of the members of the genus, their definition nevertheless can incorporate the group of yeasts which Wickerham and Burton (1954b) provisionally retained in the genus *Endomycopsis* as well as the new species under discussion.

However, as the studies of Wickerham (1951, 1957) and Wickerham and Burton (1954a, 1954b, 1956a, 1956b) have clearly demonstrated the fundamental rôle which sexuality plays in the realistic definition of a sporogenous yeast genus, the present definition of the genus *Petasospora* is incomplete and the delimitation of the species, which is to constitute the genus eventually, too vague. Until such time as the definition and delimitation of the genus *Petasospora* has been dealt with more adequately, the author favours the provisional retention of the species from the cycad strobilus in the genus *Endomycopsis*.

Superficially the new species shows some similarity with *Petasospora rhodanensis* (Ramirez et Boidin) Boidin et Abadie. The new species can, however, readily be distinguished from the *Pet. rhodanensis* by its ability to assimilate adonitol, α-methyl-D-glucoside and potassium 5-keto-D-gluconate, compounds not assimilated by the latter. The separation as a new species was further supported by the fact that interfertility between it and *Pet. rhodanensis* is absent. Active mating types obtained by Mrs. N. J. W. Kreger-van Rij and by Dr L. J. Wickerham from bisexual diploid strains, failed to react with active mating types of *Pet. rhodanensis*.

For the new species, the name *Endomycopsis wickerhamii* is proposed in recognition of Dr L. J. WICKERHAM's meritorious contributions in yeast taxonomy and in particular for having introduced heterothallism as a taxonomic tool.

Cultures of *Endomycopsis wickerhamii* have been deposited in the yeast collections of the Centraal Bureau voor Schimmelcultures in Delft and of the Northern Utilization Research and Development Division of the U.S. Department of Agriculture in Peoria.

Endomycopsis wickerhamii nov. spec.

In extracto malti, cellulae subovoidiae, longovoidiae elongataeque, (1.9 — 6.3) × (3.5 — 31.0) μ, singulae, binae aut in catenis ramosis. Mycelium verum, in diameter (2.5 — 4.8) μ. Post dies 3, pellicula non-nitida, sursum repens formatur.

Sedimentum. Post unum mensem 17° C., pellicula, sedimentum et annulus formantur.

In agar malti, cellulae subovoidiae, longovoidiae elongataeque, (1.8 — 5.0) × (2.5 — 31.0) μ, singulae, binae aut in catenis ramosis. Mycelium verum, in diameter (1.9 — 3.8) μ. Cultura (post unum mensem 17°C.) albiflava, parum nitens, in parte media acclivis cum strutura subtili. Margine piloso.

Pseudomycelium abundat.

Oriuntur asci aut per transformationem cellularum vegetarum diploidearum aut ex conjugatione inter cellulas sexus contrarii haploideas. Ascosporae pileiformes, formantur ad 4 in quoque asco. Ascosporae ex ascis celeriter liberantur.

Glucosum fermentatur at non galactosum, saccharosum, maltosum, lactosum, melibiosum nec raffinosum.

Glucosum, saccharosum, maltosum, trehalosum (exiguum), cellobiosum, melizitosum, xylosum, rhamnosum, ethanolum, glycerolum, adonitolum, mannitolum, sorbitolum, α-methyl-D-glucosidum, salicinum, kalium 5-keto-D-gluconicum, kalium D-gluconicum, calcium 2-keto-D-gluconicum (exiguum), acidum pyruvicum, DL acidum lacticum, acidum succinicum, et acidum citricum, assimilantur, et non galactosum, sorbosum, lactosum, melibiosum, raffinosum, inulinum, amylum dissolubile, L-arabinosum, D-arabinosum, D-ribosum, erythritolum, dulcitolum, kalium-natrium saccharicum nec inositolum.

Kalium nitricum et natrium nitrosum non assimilantur.

Arbutinum finditur.

Necessariae ad crescentiam sunt vitaminae externae.

Acknowledgements.

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ACTION *IN VITRO* DE LA FENACINE ALFA-CARBOXYLAMIDE SUR LES VIRUS ENCEPHALITIQUE EQUIN OUEST, POLIOMIELITIQUE, FIXE DE LA RAGE ET VACCINAL

par

A. GARCÍA GANCEDO et **GONZALO SIERRA**

(Reçu le 14 mai 1959).

INTRODUCTION.

La plupart des virus excepté les grands virus, dont le nom provient de leur grandeur et de leur complexité, restent encore inattaquables aux agents chimiothérapeuthiques.

Nous décrivons dans ce travail l'action *in vitro* de la fénacine alfa-carboxylamide sur les virus encéphalitique équin Ouest, poliomiélitique, fixe de la rage et vaccinal; ce métabolite provenant de bactérie, a été isolé de culture submergée de *Ps. aeruginosa* (SIERRA et VERINGA, 1958) comme étant l'agent responsable de l'activité antifongique observée précédemment dans le liquide métabolique de cette bactérie.

MATERIEL ET METHODES.

1. Souches utilisées. Nous avons utilisé comme microorganismes témoins des souches du virus de l'encéphalite équine Ouest (isolé par le Dr B. HOWIT en Californie); le virus poliomiélitique S K (New Haven) adapté à la souris par JUNGEBLUT. Un virus de la rage (souche conservée dans cet Institut) et un virus vaccinal provenant de la pulpe dermique de veau, soumis, pour le libérer de bactéries, à une filtration à travers une bougie de MANDLER à pores moyens selon la technique de MONTEIRO et GODINHO (1930) après l'avoir trituré avec du sable stérile dans du bouillon glucosé, à pH 8, et centrifugé. Toutes ses souches sont conservées dans la Section de Virologie de l'Institut „Jaime Ferrán".

Le virus de l'encéphalite équine Ouest est conservé par passage sur cerveau de souris, lesquels sont triturés aseptiquement et puis lyophylisés et conservés à —20°C. Le virus poliomiélitique est conservé sur cerveau de souris en glycérine (pH 8) stérile à —20°C. Le virus de la rage est conservé sur cerveau de lapin en glycérine stérile tamponnée à pH 8, à température de —20°C. Le virus vaccinal est conservé lyophylisé à —20°C.

2. Isolement de la fénacine alfa-carboxylamide. Nous avons obtenu la fénacine alfa-carboxylamide à partir de la chlororafine isolée d'une culture submergée de *Ps. aeruginosa* en milieu glycériné.

3. Animaux employés. Des souris blanches de 18—22 g, exemptes de maladies. De jeunes lapins soumis à quarantaine avant leur emploi.

4. Titration des virus. Les virus encéphalitique et poliomiélitique sont titrés sur souris selon la méthode de Reed et Muench (1938) en calculant la DL_{50} par voie intracérébrale d'échantillons de matériel virulent obtenus par des passages récents sur souris. Le virus de la rage a été titré sur lapin en faisant des dilutions doubles du matériel virulent et en inoculant des lapins intracérébralement. Le virus vaccinal a été titré en faisant des dilution décimales du matériel et en faisant des ponctions intradermiques sur le dos rasé de lapins selon la technique de Groth (1929).

5. Essai de l'activité. Nous avons préparé une suspension de matériel préalablement titré, avec le virus encéphalitique équin Ouest et le virus poliomiélitique, de façon à avoir dans 0,03 ml de cette suspension 200 SDL_{50} (doses létales pour 50% des souris). On mélange un volume de cette suspension avec un volume égal de fénacine alfa-carboxylamide en suspension 1/100; il reste dans 0,03 ml (quantité inoculée intracérébralement par souris) 100 SDL_{50} du virus et une quantité de substance diluée maintenant au 1/200. On emploie comme contrôle un autre mélange identique en remplaçant la substance essayée par un volume égal de solution saline physiologique. Les deux mélanges sont stockés au frigidaire à 4°C. durant 5 heures. On inocule intracérébralement deux groupes de souris avec l'un ou l'autre des mélanges.

Dans les épreuves avec le virus rabique on prépare une suspension de virus à 1/5 qu'on mélange à un volume égal d'une solution à 1/100 de la substance; le virus est donc dilué à 1/10 et la substance à 1/200 (le virus rabique tue tous les lapins, à cette concentration, au bout de sept jours après l'inoculation selon la détermination par titration préalable). On a preparé aussi le mélange contrôle avec le virus à 1/5 et la solution saline. La période d'incubation à 4°C., a été la même que celle utilisée avec les autres virus. On inocule avec chacun des mélanges un groupe de lapins.

Nous préparons une suspension de matériel virulent avec du virus vaccinal, titré à 1/25, lequel est mélangé à un volume égal de substance dilué à 1/100, ce qui dilue le premier à 1/50 et le second à 1/200. De même que dans les cas précédents, nous faisons un autre mélange de virus à la même dilution avec une solution saline stérile comme témoin. Les mélanges sont stockés à la même température et durant 5 heures comme dans les cas précédents. Puis l'on fait des ponctions intradermiques sur lapins, selon la technique de GROTH en inoculant 0,20 ml du mélange par ponction sur le dos rasé des lapins. On fait trois ponctions par lapin; une avec le mélange de virus, une autre avec le mélange virus-solution saline et une autre avec la substance.

6. Essai de la toxicité. Nous avons inoculé par voie intrapéritonéale et souscutanée des groupes de souris avec 0,25 ml d'une solution saline de la substance, et par voie intracérébrale avec 0,03 ml. Nous avons inoculé des lapins avec 2,0 ml par voie souscutanée et intrapéritonéale et 0,15 ml par voie intracérébrale.

RESULTATS.

Les souris inoculées avec le virus encéphalitique équin Ouest et une solution saline (contrôle) meurent, tandis que celles inoculées avec le mélange virus-fénacine alfa-carboxylamide ne montrent aucun symptôme de maladie pendant les 26 jours d'observation après l'inoculation. Ces souris sont réinoculées intracérébralement avec 100 SDL_{50} du même virus. Toutes meurent alors.

La fénacine alfa-carboxylamide n'a aucune action sur les virus poliomiélitique, rabique et vaccinal.

La substance n'est pas toxique pour la souris en doses de 500 mg par kg par voie intrapéritonéale; de 0,015 mg/kg intracérébralement et de 0,123 mg/kg par voie souscutanée.

Les lapins supportent les doses essayées de 0,0015 mg/kg intracérébralement, de 0,03 mg/kg intrapéritonéalement et de 0,02 mg/kg par voie souscutanée, en inoculant des doses par les deux dernières voies pendant sept jours consécutifs.

DISCUSSION.

Les résultats obtenus „*in vitro*" avec la fénacine alfa-carboxylamide sur le virus encéphalitique équin Ouest peuvent être intéressants, car il s'agit d'une inhibition d'un petit virus (30 mμ).

La substance semble avoir une certaine action spécifique, puisqu'elle n'agit pas sur les autres virus essayés. Ce fait, outre son innocuité, semble indiquer que l'action de la fénacine alfa-carboxylamide n'est pas due à une dénaturalisation des protéines. Elle peut être réellement due à une inhibition de la multiplication du virus, ne produisant donc pas la maladie et ne conférant pas d'immunité, ainsi que montre le fait que la réinoculation des souris survivant à la première inoculation avec le mélange fénacine alfa-carboxylamide-virus encéphalitique (100 SDL_{50}) avec une dose égale de virus entraîne leur mort.

Nos recherches seront continués afin d'élucider ces problèmes et l'action „*in vivo*" de cette substance.

Sommaire.

Nous avons trouvé que la fénacine-alfa-carboxylamide (métabolite de *Ps. aeruginosa*) inhibe „*in vitro*", le virus encéphalitique équin Ouest, tandis qu'il n'exerce aucune action observable sur d'autres virus essayés. La substance n'est pas toxique pour la souris et le lapin, dans les concentrations et par les voies utilisées.

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THE GENUS *ACETOMONAS*

by

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(Received May 25, 1959).

INTRODUCTION.

It is now known, through the work of LEIFSON (1954), confirmed by SHIMWELL (1958), that the acetic acid bacteria do not comprise, as previously believed, a homogeneous group classifiable in a single genus *Acetobacter* in the family *Pseudomonadaceae* on account of the alleged possession, if motile, of polar flagella. LEIFSON has shown that there are two biochemically and morphologically distinct (and therefore probably phylogenetically unrelated) types of acetic acid bacteria. He has proposed that the genus *Acetobacter* should be retained and reserved for those bacteria which oxidize alcohol through acetic acid to CO_2 and H_2O and which, if motile, have peritrichous flagella (not polar ones as previously stated in the literature). For those which do not oxidize alcohol further than acetic acid, and which, if motile, have polar flagella, he has proposed the appropriate generic name *Acetomonas*. *Acetobacter*, by virtue of its peritrichous flagellation, should be removed from *Pseudomonadaceae*, whilst *Acetomonas* must obviously remain therein.

The genus *Acetobacter*, as redefined by LEIFSON, has recently been studied and re-assessed by one of us (SHIMWELL, 1959) and need not be further reviewed here. The present paper is concerned with the non-acetate-oxidizing genus *Acetomonas*.

HISTORICAL.

The first acetic acid bacterium incapable of oxidizing alcohol further than acetic acid was described by HENNEBERG (1897) under the name *Bacterium oxydans*. It was particularly active towards sugars, the oxidation of ethyl alcohol being relatively less pronounced than with most (but not all) other types of acetic acid bacteria, rarely exceeding about 2.7% acetic acid, whereas selected strains of acetic acid bacteria found in vinegar and vinegar generators could sometimes produce up to 8% and occasionally more.

In addition to emphasizing the non-oxidation of acetic acid, HENNEBERG also particularly commented on the type of motility observed. This he described as apparently due to polar flagella on account of the direction of cell-motion. He did not, however, demonstrate the type of flagellation by staining. As HENNEBERG went out of his way to mention this probably polar flagellation, and as he did not stress the probable type of flagellation of other species, he seemed to think it notable and perhaps novel. Further comment on his full description of *B. oxydans* will be made later.

In the following year HENNEBERG (1898) described another similar non-over-oxidizing species which attacked precisely the same sugars and alcohols as *B. oxydans*, but differed from that species in rarely giving rise to involution forms, and in producing intense viscidity in liquid media containing dextrin. He regarded this as a separate species under the name *Bacterium industrium.*

BEIJERINCK (1911) isolated from beer another non-acetate-oxidizing acetic acid bacterium which produced, in addition to acid, a brown to black pigment in peptone and similar media containing either glucose or maltose.The colour-intensity of the pigment (which, it is important to note, was water-soluble) varied greatly from strain to strain. He also stressed the rapid oxidation of mannitol and sorbitol respectively to fructose and sorbose. BEIJERINCK named this species *Acetobacter melanogenum.* The further detailed description given by BEIJERINCK will also be discussed later.

BAKER, DAY and HULTON (1912) made a technological study of an acetic acid bacterium producing ropiness in beer. This organism they named *B. aceti viscosum.* It did not over-oxidize acetic acid and the nature of the constituent of beer from which the organism made the slime could not be discovered. Nor did they associate their organism with *Bacterium industrium* Henneberg.

KLUYVER and DE LEEUW (1924) described a species also not oxidizing acetic acid or acetate, and producing much gluconic acid from glucose, and dihydroxyacetone from glycerol. They named this organism *Acetobacter suboxydans.* In later years this name has become very popular, completely obscuring the priority of HENNEBERG (1897).

who was the first to isolate and describe non-acetate-oxidizing acetic acid bacteria. Cultures stated to be "*A. suboxydans*" have become perhaps the most extensively used of all acetic acid bacteria in biochemical studies.

SHIMWELL (1936) isolated from beer the organism responsible for the ropiness thereof. It somewhat resembled the earlier *B. aceti viscosum* of BAKER, DAY, and HULTON, and the starting material from which the slime was produced was later identified (SHIMWELL, 1947) as the residual dextrin in beer. SHIMWELL did not, at that time, associate his "*A. capsulatum*" with HENNEBERG's *B. industrium.*

FRATEUR (1950) recognised that those acetic acid bacteria failing to over-oxidize acetic acid were different from others, but considered that taxonomically this property was more one of degree rather than absolute. He classed such bacteria in his "suboxydans group" as distinct from his (over-oxidizing) "mesoxydans group" (defining them both, however, as ketogenic). He recognised two non-overoxidizing species, namely *A. suboxydans* and *A. melanogenum,* with several varieties of each.

LEIFSON (1954) showed, for the first time, that the non-overoxidizing strains not only differed from other acetic acid bacteria in the type of their oxidative attack on alcohol, but were also morphologically entirely different types of bacteria. This has already been referred to under "Introduction". LEIFSON's flagella photomicrographs proved unacceptable to the editors of BERGEY's Manual (1957), who awaited electron micrographs before accepting LEIFSON's findings.

SHIMWELL (1958) confirmed LEIFSON's work, and published photomicrographs of the peritrichous flagellation of LEIFSON's amended *Acetobacter* genus, and of the polar flagellation of his new *Acetomonas* genus. He also published an electron micrograph of his *A. rancens* strain, confirming the peritrichous flagellation of *Acetobacter* proper (by generous permission of Dr. JAMES M. SHEWAN, of Torry Research Station, Aberdeen, where the micrograph was taken).

ASAI and SHODA (December, 1958) rejected LEIFSON's *Acetomonas* on the alleged grounds that such bacteria "are scarcely able to oxidize ethanol to acetic acid". They suggested the genus "*Gluconobacter*" instead. They also (wrongly) implied priority over SHIMWELL in showing that some strains were monotrichous and that not all were multitrichous, as stated by LEIFSON. SHIMWELL's publication was dated March 1958.

DEFINITIONS.

"Over-oxidize". This technical vinegar-maker's term is here occasionally used for the sake of brevity, to mean that alcohol is not only oxidized to acetic acid, but that the latter is then further oxidized to CO_2 and H_2O.

"Ketogenic". This is used, for simplicity, to denote the production of dihydroxyacetone from glycerol, as this is virtually the sense in which it is used by FRATEUR (1950). "Ketogenic" acetic acid bacteria may also produce 5-ketogluconic acid from glucose, fructose from mannitol, and so on. The production of 2-ketogluconic acid is a property probably common to most acetic acid bacteria except FRATEUR's "peroxydans group" (FRATEUR, SIMONART and COULON, 1953). It was not used, however, as a criterion in his classification, and is not so used in the course of this paper.

"Suboxydans Group". This was the term given by FRATEUR to the 4th of his 4 groups (the highly ketogenic, non-overoxidizing ones). In this paper "suboxydans group" only denotes the lack of over-oxidation of alcohol to CO_2 and H_2O. In our opinion this property alone probably suffices to separate such acetic acid bacteria from all others, as will be seen.

Specific Names. Although we finally propose, after LEIFSON (1954), that "*Acetomonas*" should be adopted henceforth as the name of the genus of non-overoxidizing acetic acid bacteria, the original names of existing species have been retained throughout this paper until the diagnosis of the genus *Acetomonas* comes to be given at the end. This is to enable such species to be identified and linked with their original creators.

METHODS.

In the main these have been those used by FRATEUR (1950), but have been combined with others. In addition, feeble or doubtful oxydogram results have been augmented by growing the strains on slopes of the same media for a longer period. The all-important criterion of non-overoxidation of acetic acid or acetate has been given particular attention, and has been checked by a variety of methods by both of us separately; *e.g.*, in addition to yeast-extract (YE)-alcohol-$CaCO_3$-agar oxydograms and slopes, slopes of YE-alcohol-agar, without $CaCO_3$ but with brom-cresol-green as internal indicator, have been used, as have also cultures in liquid media.

Flagella staining has been by SHIMWELL's (1958) modification of FISHER and CONN's method. Electron micrographs have also been made of some doubtful strains. The presence or absence of visible motility has in all cases been determined in hanging drop, for, as HENNEBERG (1897) and FRATEUR (1950) both noted, motility under a cover-slip may sometimes cease in a matter of seconds, owing to the absence of air.

Pigment production, the main (perhaps only) difference between *A. suboxydans* and *A. melanogenum* in FRATEUR's system, has been mainly studied on YE-agar media containing 10% of the particular sugar and 3% $CaCO_3$. Lower and various concentrations of sugar and $CaCO_3$ have also been used additionally in certain cases.

Colony form has been examined under a magnification of × 40, using a × 4 objective and a × 10 eyepiece, by transmitted light or oblique transmitted light (SHIMWELL, 1957, 1958, 1959).

Cultures have been maintained on agar slopes of either unhopped beer agar (in the case of beer strains) or apple juice agar (in the case of cider strains), both incubated at 26°C. until growth appeared, thereafter at room temperature or at 10°C. in the refrigerator. Most strains do not remain viable for long on these media if continuously incubated at 26°C. Freeze-drying has proved very satisfactory.

EXPERIMENTAL.

In all, 50 strains have been studied. Most of these were isolated from beer, brewery yeast, fermenting brewery wort, apple juice, fermenting apple juice, and cider. In addition, strains and varieties labelled *A. suboxydans* and *A. melanogenum* have been obtained from culture collections. Particular care has been taken to obtain the authentic strain of *Acetobacter suboxydans* Kluyver and de Leeuw. Most of the strongly pigmented strains have been isolated from apple juice and cider, in which these types proved plentiful. It is believed that the strains of FRATEUR's varieties of *A. suboxydans* and *A. melanogenum* are his authentic ones. All cultures from collections were carefully typed before the label was accepted, as were also our own isolates. A surprisingly large number of strains labelled "*A. suboxy-*

dans" from collections were found to be overoxidizing *Acetobacter* species and were rejected. It has become clear, indeed, that in some widely cited investigations the "*A. suboxydans*" used was not of this type at all.

Criteria for Differentiation of Species and Varieties.

For this purpose FRATEUR used the production of acid and/or pigment and/or mucilage on glucose, sucrose and maltose. All strains producing a brown to black pigment on YE-glucose-$CaCO_3$-agar were identified as *A. melanogenum* or as a variety of that species. Those not producing pigment on that medium he identified as *A. suboxydans* or as a variety of that species. It is important to note, however, that his *A. suboxydans*, and all its varieties, did produce a brown pigment if fructose was substituted for glucose. Thus the difference between the two species was really a matter of on which of these sugars the pigment was produced.

We have adopted the same criteria, but with the addition of fructose, in attempting to differentiate our strains. In addition to the species and varieties observed by FRATEUR a number of other combinations of acid, pigment, and mucilage production on the four sugars have been found. Altogether the strains in our collection have fallen, so far, into one or another of 13 groups with different combinations of FRATEUR's criteria, *i.e.* 6 more than the 7 of FRATEUR. In Table 1 these 13 different combinations are listed together with their specific or varietal names (if any) whilst it is also indicated in the first column whether the combination of properties found correspond with the description of the creator of the particular species or variety concerned.

We think it likely that if an even larger collection were to be studied further combinations of the criteria might be encountered. Thus the picture presented begins to resemble that found by SHIMWELL (1957, 1958) in the case of *Acetobacter* proper.

A word is necessary about the significance of "P" in the table, where it indicates water-soluble pigment production. The colour and intensity of the pigmentation varied greatly between all our own strains, and amongst named species and varieties also, varying from pale brown to darker brown to black, if one allowed incubation to proceed until the maximum colour intensity had developed; this often required 3 or 4 weeks or more at 26°C. In addition, the partly

TABLE 1.

Acid, Pigment, and Mucilage Production.

Characters correspond to:	Glucose	Fructose	Sucrose	Maltose
1. *A. suboxydans* (authentic strain)	AP	AP (pale)	O	AP
2. *A. suboxydans* (FRATEUR's description)	A	AP	O	A
3. *A. suboxydans* (N.C.I.B. 3734)	A	A	O	A
4. *A. suboxydans* var. *muciparum*	A	AP	AM	A
5. *A. suboxydans* var. *biourgianum*	A	AP	AM	O
6. *A. suboxydans* var. *hoyerianum*	A	AP	O	O
7. *A. melanogenum* (BEIJERINCK's description)	AP	O	O	AP
8. *A. melanogenum* (FRATEUR's description)	AP	AP	O	O
9. *A. melanogenum* var. *maltovorans*	AP	AP	O	AP
10. *A. melanogenum* var. *malto-saccharovorans*	AP	AP	AP	AP
11. *A. capsulatum* (N.C.I.B. 4943)	AP	AP	O	A
12. *A. melanogenum* (our strain C. 1)	AP	AP	AMP	O
13. *A. suboxydans* (our strain C1A)	A	AP	AM	O

A = acid; P = pigment; M= mucilage; O = no action.

developed pigmentation was sometimes "plain" brown, sometimes orange-brown, and sometimes orange-red; the black final colour produced by some strains appeared to be largely a very deep red. To have indicated this in the table would have complicated it unduly; "P" merely means, therefore, that some definite degree of pigmentation developed, without indicating its intensity of colour. It may be mentioned, however, that the colour produced by the authentic (supposedly colourless) strain of *A. suboxydans* was by no means the palest, being red-brown on glucose and fructose, and orange-brown on maltose, after 4 weeks at 26°C.

Ketogenic Power.

All our strains, except one, produced dihydroxyacetone from glycerol. The one exception was, surprisingly, a strain stated to have been deposited by KLUYVER himself in the National Collection of Type Cultures (N.C.T.C.) in 1932. This strain is now No. 3734 in the National Collection of Industrial Bacteria (N.C.I.B.).

No dihydroxyacetone-production was detectable with this strain, nor did it produce fructose from mannitol. Mr. K. R. BUTLIN, however, (private communication) says that when he studied this strain in 1934 it produced much dihydroxyacetone from glycerol (see also SHIMWELL, 1958).

This strain is also the only one in our collection which has not produced any signs of pigment on any sugar. The authentic strain of *A. suboxydans* from Delft, however, is both ketogenic and pigment-producing, which suggests that if N.C.I.B. 3734 and the Delft culture were originally of the same strain, the former has now become replaced by colourless non-ketogenic mutants, a supposition supported by the finding of SHIMWELL (1957) and by the fact that both strains still produce acid from the same sugars.

DISCUSSION.

The data in Table 1 together with our other observations, require some discussion to bring out their taxonomic significance. A surprising result is that the authentic *A. suboxydans* strain produces brown pigment from glucose, and is therefore virtually classifiable as *A. melanogenum* or a variety thereof. It is also to be noted that its production of acid is on the same sugars as with *A. capsulatum*, (which is similarly strongly ketogenic).

We have found, however, that this *A. suboxydans* strain differs from *A. capsulatum* in two other characters studied. It freely produces hypertrophied cells and does not make dextrin media viscid. *A. capsulatum* rarely produces hypertrophied cells and does produce viscidity in beer-dextrin media. These two differences are precisely the same as those emphasized by HENNEBERG in the case of his *B. oxydans* and *B. industrium* respectively; he particularly stated that the sugar oxidation of his two species were identical. He did not study pigment production on sugar-chalk media.

Ketogenic power, extensively used by FRATEUR in his system, was not specifically recorded by HENNEBERG; he merely stated that glycerol (and mannitol) were "oxidized". KLUYVER and DE LEEUW, however, noted it in the case of their *A. suboxydans*. On the other hand, KLUYVER and DE LEEUW did not use some of HENNEBERG's criteria. Where criteria are common to both investigations, however, they closely correspond. We feel, in view of the above, that *A. suboxydans* was probably a later isolate of *B. oxydans* Henneberg, and *A. capsulatum* of *B. industrium* Henneberg (see Table 2).

TABLE 2.

	B. oxydans (HENNEBERG'S description)	*A. suboxydans* (authentic strain)	*B. industrium* (HENNEBERG'S description)	*A. capsulatum* (authentic strain)
Oxidation of acetic acid	—	—	—	—
Gluconic acid from glucose	+	+	+	+
Gluconic acid from maltose	+	+	+	+
Action on glycerol	"oxidized"	dihydroxyacetone	"oxidized"	dihydroxyacetone
Pigment	N	N	N	N
Cell-chains	common	common	rare	rare
Hypertrophied cells	common	common	rare	rare
Ropiness in beer dextrin	—	—	+	+

N = not originally studied.

Production of Water-Soluble Pigment from Glucose.

This criterion was used by BEIJERINCK (1911) to establish his *Acetobacter melanogenum*, and maintained by FRATEUR (1950) for its differentiation from *A. suboxydans*. However, quite apart from the fact that there seems no reason why pigment production on glucose should be regarded as so much more important than similar pigment production on fructose, maltose, and sucrose, it seems to have been overlooked that BEIJERINCK himself (1911) wrote (page174), "Strains of varying pigment producing potency were isolated from various beers . . .". And, on page 175, "Long-maintained cultures show two main types of variability. They can completely lose the power of producing pigment . . . also their usually slight power of slime formation can considerably increase". He added that, when having lost pigment production, such strains became indistinguishable from colourless forms. Thus it seems that the specific insignificance of pigment production on any particular sugar, brought out in Table 1, was implicit in BEIJERINCK's original work of 1911.

The probable mechanism of such loss of pigmentation has recently been indicated by SHIMWELL (1957) who found that a strain of *A. melanogenum* (the only one thus examined) gave rise to two colony forms, one pigment-producing and the other not. Moreover, the colourless mutant could regain pigment production, simultaneously regaining its original colony form (Fig. 1).

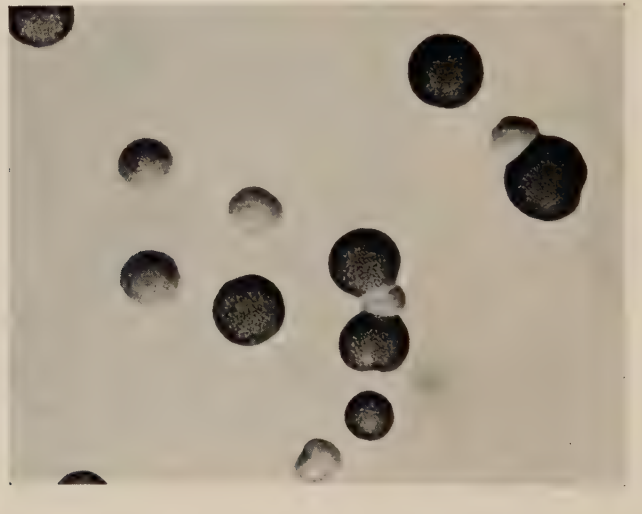

Fig. 1. *A. melanogenum* N.C.I.B. 8086.
Pigmented and non-pigmented colonies. × 40.

Finally the remaining results reported in Table 1 need perhaps a further word. Those familiar with the organisms concerned, and with the appropriate literature, will notice that the results recorded in Table 1 sometimes conflict somewhat with the statements of others. We have recorded the results of our work, however, and where such confliction was encountered the tests were repeated several times.

CONCLUSIONS.

Apart from the fact that our study of the literature, and our experimental results, together with those of SHIMWELL (1958), show that LEIFSON's differentiation of *Acetomonas* from *Acetobacter* is fundamentally essential and should be accepted, we consider that the specific distinction between "*A. suboxydans*" and "*A. melanogenum*" is based on criteria (e.g. pigment and/or acid production on a particular sugar) too slight, too variable from strain to strain, (and probably mutable within a strain) to be worthy even of varietal status. There appears, therefore, to be no alternative to limiting the number of species to one. To do otherwise would necessitate innumerable species based on variable and/or mutable criteria.

TAXONOMY.

Acetomonas Leifson amended Shimwell.

Diagnosis: Bacteria oxidizing ethyl alcohol to free acetic acid, but not further to carbon dioxide and water. Motile or non-motile. If motile the cells possess one or more polar flagella.

Type (and sole) species:
Acetomonas oxydans (Henneberg) Shimwell and Carr.
Diagnosis: The same as that of the genus.

We consider that the above generic and specific diagnoses are adequate. The inclusion of other and variable or mutable properties which "may or may not" be possessed, such as pigment on glucose and oxidation of this or that substrate, are mere varietal or strain characteristics, not only unnecessary in the present context, but likely to lead to excessive future "splitting" into innumerable unstable "varieties".

Some such minor properties (for instance the production of viscidity in beer containing dextrin) may be of considerable technological, but scarcely of taxological, significance. The latter is the question we are here considering.

Nor do we think it necessary to specify that the cells should even be rod-shaped, gram-negative, or catalase-positive, although the strains we have studied possessed these properties. Some strains of "*A. capsulatum*" however, when growing in beer or malt extract, are almost coccoid; in fact they have at times been microscopically mistaken for cocci by brewery technologists. Similarly catalase-positivity was formerly claimed for all acetic acid bacteria, but it has since been found that many strains of *Acetobacter* proper are completely devoid of catalase, although strictly aerobic. The diagnoses of both *Acetomonas* and *A. oxydans*, although brief, are considered sufficiently exclusive to admit only bacteria of the type under discussion.

Strain Variations.

The following characteristics, amongst others, have been found to vary from one strain to another (and often even within the same strain): Cell-shape and size; motility; number of flagella per cell; colony form and other cultural characteristics; pigment production on glucose, fructose, maltose, or sucrose; intensity of ketogenic power; slime production; and relatively small variations in apparent optimum temperature (as the medium used, and its acidity and/or pH, may affect this, as may also the definition of "optimum").

Type Culture.

As far as we can discover, no authentic cultures of Henneberg's original isolate of *B. oxydans* exist today, although some strains with this label are to be found in culture collections. Furthermore, we have not encountered any strains in which every characteristic, morphological, cultural, biochemical, and technological is identical with those described in detail by Henneberg (1897). As such absolute identity, however, rarely (if ever) occurs in nature, we consider that this does not prevent the establishment of a neotype. We are therefore depositing with the N.C.I.B. two cultures which closely correspond to Henneberg's description of *B. oxydans* except in respect of one sugar fermentation in each case. The first culture (from beer) differs from *B. oxydans* (and/or *B. industrium*) in that it does not acidify sucrose; the second (from cider) does acidify sucrose but does not acidify maltose, phenomena probably associated with their respective isolation from a sucrose-free and a maltose-free habitat. An electron micrograph of the cider strain is shown in Fig. 2, where it will be seen that one cell has 5 polar flagella and another a single flagellum, in accordance with the diagnosis of Leifson (1954) as amended by Shimwell (1958).

"*Acetimonas*" Orla-Jensen.

In 1909, Orla-Jensen, in the course of propounding a new bacteriological classification, coined many new generic names ending in

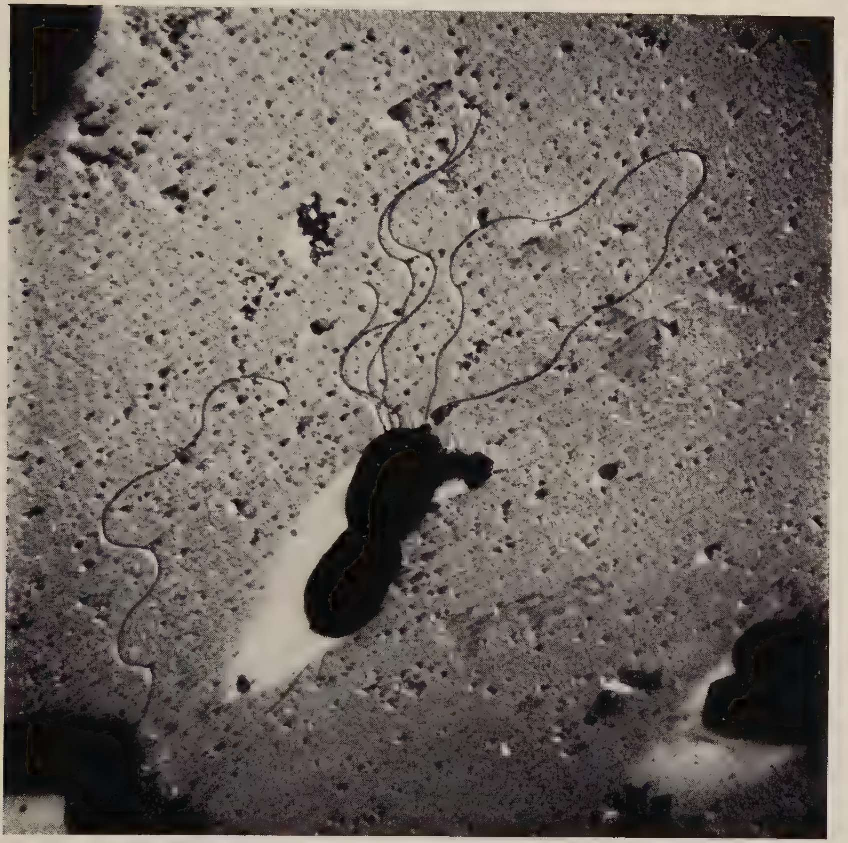

Fig. 2. *Acetomonas oxydans* (cider strain).
Electron micrograph.

"monas". For the acetic acid bacteria as a whole he proposed "*Acetimonas*" to indicate the alleged polar flagellation of this group.

We submit that this name must be rejected because (1) He did not describe the genus so named. (2) He did not designate a type species. Indeed the only species mentioned was *A. schützenbachi*; this, however, was a non-motile species, and, even if it had been motile, should have had peritrichous flagella, as it overoxidized alcohol to CO_2 and H_2O (Henneberg, 1926; Leifson, 1954).

As Orla-Jensen's "*Acetimonas*", therefore, was not only superfluous, but also terminologically wrong and inadequately described, there can be no grounds for considering that mere similarity in spelling to the properly described and published *Acetomonas* Leifson justifies any priority, particularly as this would only perpetuate the former belief that all motile acetic acid bacteria have polar flagella.

Gluconobacter Asai and Shoda.

This must be rejected for a number of reasons. The statement that "*Acetomonas*" is "unreasonable", because its strains "scarcely" acidify alcohol at all, obviously conflicts with the fact that until LEIFSON's work they were not differentiated from *Acetobacter* species, being recognised by all as typical acetic acid bacteria. ASAI and SHODA's definition of *Gluconobacter* describes acetic acid production from ethanol as "weak or none". Such bacteria giving "none" would be *Pseudomonas* strains, however, excluded from *Acetomonas* by definition. Apparently these authors' collection included both *Acetomonas* and *Pseudomonas* strains.

The bacteria dealt with in our present paper are, in contrast (like those of HENNEBERG, BEIJERINCK, KLUYVER and DE LEEUW, SHIMWELL, FRATEUR, and LEIFSON) acetic acid bacteria, thriving (usually preferentially) in acid media, and not bacteria with merely somewhat similar oxidative properties on substrates other than ethanol in media of near-neutral reaction.

ASAI and SHODA have also included in their genus *Gluconobacter* several strains which they state have polar flagella and yet (surprisingly) oxidize acetate to carbonate. Their electron micrographs appeared somewhat inconclusive, however, so by courtesy of Dr T. ASAI we have obtained and studied these strains of *G. melanogenus*. We find them to possess not polar, but peritrichous flagella. These are sparse (1 to 4) and become readily detached, so that some cells appearing monotrichous were perhaps really multitrichous before staining (Fig. 3). These acetate-oxidizing organisms are therefore *Acetobacter* strains, LEIFSON's generic criteria thus remaining undisturbed.

Acetobacter Beijerinck.

We draw attention to the fact that before LEIFSON's discovery of the dual composition of the previously defined genus *Acetobacter*, all published descriptions of its properties were incorrect, for they consisted of a single unified diagnosis of two morphologically, biochemically, (and therefore probably phylogenetically) different types of bacteria.

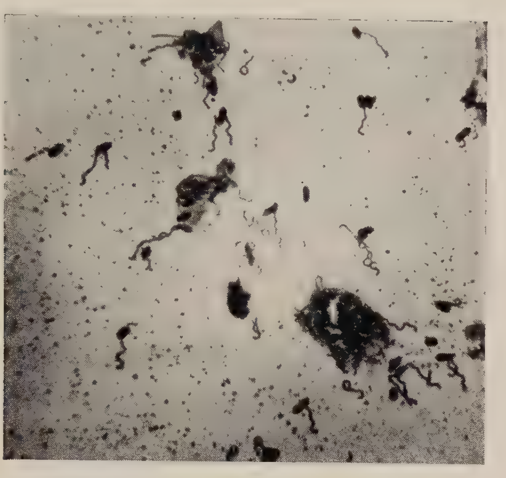

Fig. 3. Sparse peritrichous flagella of *"Gluconobacter melanogenus"*. Also detached flagella. × 1000.

The description of a genus, to be valid, must not only be published, but surely must also be correct. This combination of requirements is fulfilled only by *Acetobacter* as described by LEIFSON (1954). It therefore seems that not only *Acetomonas*, but also *Acetobacter*, should probably be attributed to LEIFSON.

Summary.

A study of 50 strains of acetic acid bacteria incapable of further oxidation of acetic acid, together with a study of the literature, has led to the conclusion that the segregation of such bacteria in a new genus *Acetomonas* Leifson amended Shimwell is fundamentally and phylogenetically sound and should be accepted.

No objective grounds justifying the specific separation of pigment-producing strains from alleged colourless ones have been discovered, pigmentation on a particular sugar varying from strain to strain, and also (probably by mutation) within a single strain. Similar arguments apply to the other biochemical and morphological criteria studied. A single species *"Acetomonas oxydans* (Henneberg) Shimwell and Carr" is therefore proposed and diagnosed. It is considered that *A. suboxydans* Kluyver and de Leeuw is a synonym of, and a later isolate of, *Bacterium oxydans* Henneberg (1897).

It is suggested also that the previous necessarily inaccurate des-

cription of *Acetobacter* Beijerinck may invalidate the claims of anyone before LEIFSON to have originated, or properly described, this genus, and that "*Acetobacter* Leifson" may now be the correct designation.

"*Acetimonas*" Orla-Jensen, and "*Gluconobacter*" Asai and Shoda are rejected, and the reasons given.

Acknowledgements.

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ETUDE IMMUNOLOGIQUE DE SERUMS DE MOUTON ANTIDIPHTERIQUES

par

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avec la collaboration technique de Mademoiselle O. Bepoldin et Monsieur W. J. van Rhee

(Reçu le 1er juillet, 1959).

INTRODUCTION.

La mobilité électrophorétique des constituants sériques a déjà fait l'objet de nombreuses études. Les anticorps ont en général été trouvés associés aux gamma-globulines des sérums de diverses espèces animales. Une exception à cette règle a entre autres été observée chez le cheval où les anticorps ont été trouvés dans la fraction gamma-globulines ou dans la fraction ayant une mobilité intermédiaire entre celle des beta et des gamma-globulines. Cette fraction a reçu diverses désignations : globulines-T, beta-2 ou gamma-1 (BOYD, 1956; CINADER et WEITZ, 1950, 1953; DEUTSCH et NICHOL, 1948; KECKWICK, 1941; KECKWICK et RECORD, 1941; VAN DER SCHEER *et al.*, 1940a et b, 1941a, TISELIUS et KABAT, 1939). Dans des cas plus rares on a trouvé des anticorps dans les alpha-globulines (RAYNAUD *et al.*, 1959b).

Les anticorps se trouvent chez le cheval, au début de l'hyperimmunisation, souvent associés de façon prédominante à la fraction gamma-globulines; tandis qu'en fin d'hyperimmunisation, on les trouve à concentration élevée dans la fraction beta-2-globulines (KECKWICK, 1941; KECKWICK et RECORD, 1941; RAYNAUD *et al.*, 1959a; RELYVELD *et al.*, 1957a, 1957b, 1958a, 1958b).

L'étude électrophorétique du sérum de mouton normal a déjà été faite par certains auteurs. La composition du sérum normal a été déterminée par électrophorèse sur papier par GEINITZ (1954).

Uriel *et al.* (1957) ont employé la technique d'électrophorèse en gélose et noté la présence de deux gamma-globulines.

Nous présentons ici les résultats de l'étude électrophorétique du sérum de mouton antidiphthérique et ceux de l'étude quantitative de la réaction toxine-antitoxine de mouton. Nous avons dans ce but eu recours aux techniques de l'immuno-électrophorèse et de l'électrophorèse en gélose. Les techniques de double diffusion en gélose nous ont renseignés sur la présence d'anticorps autres que l'antitoxine diphtérique. Nous avons employé une toxine brute concentrée et une toxine pure pour les mettre en évidence.

La toxine brute concentrée contient, outre la toxine diphtérique, de nombreux autres antigènes dont nous avons déjà démontré l'existence (Relyveld *et al.*, 1954, 1956, 1958b). Nous avons nommé ces antigènes "antigènes accessoires". L'emploi d'une toxine brute pour l'hyperimmunisation entraîne la formation d'anticorps dirigés spécifiquement contre les antigènes accessoires. Nous avons nommé les anticorps qui ne sont pas dirigés contre l'un des motifs antigéniques de la toxine mais contre d'autres protéines présentes dans le filtrat de culture "anticorps accessoires" (Raynaud *et al.*, 1959a, Relyveld *et al.*, 1957b).

Les impuretés présentes dans les préparations de haute pureté, même sous forme de trace, peuvent, comme nous l'avons déjà observé, donner naissance à la formation d'anticorps accessoires. Le nombre d'anticorps accessoires présents dans les sérums préparés avec une toxine brute est très élevé (au moins 15) (Raynaud, 1959d; Relyveld, 1958b).

Nous avons fait l'étude quantitative d'antitoxines de mouton brutes et purifiées. Pour étudier la réaction entre la toxine et les anticorps spécifiquement dirigés contre cette molécule, nous avons employé une toxine pure cristallisée; pour étudier l'influence des anticorps accessoires, nous avons de nouveau employé une toxine brute. La composition moléculaire antitoxine/toxine tout au long de la courbe de précipitation quantitative a été déterminée à l'aide d'une toxine pure marquée à l'iode 131.

L'étude quantitative de la réaction entre une protéine et l'antisérum correspondant de mouton n'a, à notre connaissance, jamais été faite. Nous rappelerons d'abord brièvement les résultats obtenus avec les anticorps d'autres espèces animales.

Les courbes de précipitation quantitative ont été désignées comme étant de type précipitine (type lapin), ou de type floculation (type cheval). La courbe de type lapin a une forme arrondie et passe par l'origine (le précipité formé est insoluble dans un excès d'anticorps) et a été retrouvée chez l'homme ainsi que chez la plupart des espèces animales. Les courbes de type cheval ont une forme particulière; elles présentent une zone rectiligne et le précipité est soluble dans un excès d'anticorps. Nous avons démontré (Raynaud, 1959d; Relyveld *et al.*, 1957a, 1958a, 1958b, 1959a), que la forme de la courbe de précipitation quantitative n'est pas déterminée par l'espèce animale mais est en rapport avec la mobilité électrophorétique des anticorps. Les gamma-globulines donnent une courbe de type précipitine et les beta-2-globulines une courbe de type cheval. On obtient une courbe de type lapin si la précipitation quantitative a été faite avec la fraction anticorps gamma-globulines de cheval. Il a été observé que les gamma antitoxines de cheval présentaient des rapports in vivo/in vitro élevés, en général proches de 2, tandis que les anticorps de type beta-2-globulines avaient des rapports voisins de 1.

Les études concernant ces deux types d'anticorps avaient été faites avec des saignées de chevaux spécialement sélectionnés pour la production d'antitoxine, présentant une immunité de base avant le début de l'hyperimmunisation (chevaux à Schick négatif). L'étude de la formation d'anticorps chez des chevaux sans immunité de base a montré que les deux types d'anticorps précipitants peuvent aussi avoir des rapports in vivo/in vitro très bas. Les anticorps ont dans ce cas un pouvoir précipitant assez élevé, tandis que le pouvoir neutralisant vis-à-vis de la toxine est très faible (Raynaud *et al.*, 1959a).

La réaction de précipitation quantitative entre la toxine et ces anticorps est dans ce cas analogue à la réaction de certains systèmes enzyme-antienzyme; tous les précipités formés peuvent être toxiques (Relyveld et Raynaud, 1959a).

Nous avons pu montrer que les anticorps qui apparaissent chez le cheval après immunisation par une anatoxine de haute pureté sont hétérogènes: ils sont constitués par un mélange d'au moins trois anticorps de spécificité différente, chacun d'entre eux étant produit par un motif antigénique particulier présent sur la molécule de toxine.

La mise en évidence de ces divers types d'anticorps a été réalisée à l'aide d'une toxine partiellement digérée par une protéase. Des observations analogues ont été faites antérieurement par Lapresle *et al.* (1955a, 1955b, 1957a, 1957b, 1958, 1959) pour le système albumine humaine – sérum de lapin antialbumine humaine et par Pope (1954, 1957) et Pope et Stevens (1958a, b, c) pour le système toxine – antitoxine diphtériques. Mais cet auteur a fourni de ces résultats une interprétation très différente de celle de Lapresle et de nous-mêmes (Raynaud, 1959d ; Raynaud et Relyveld, 1959c ; Relyveld, 1958b ; Relyveld et Raynaud, 1959a).

MATÉRIEL ET MÉTHODES.

1. Antitoxines étudiées.

La purification des sérums a déjà fait l'objet d'une publication détaillée (van Triet, 1959). Les moutons ont été vaccinés avec une anatoxine purifiée et adsorbée (P.T.), 15 jours avant l'hyperimmunisation proprement dite. Cette dernière a été faite avec l'anatoxine brute, ensuite avec une toxine brute partiellement détoxifiée et finalement avec une toxine brute adsorbée sur alun.

Notre étude a porté sur les sérums n° 5, bruts et digérés. Le sérum n° 5 était un mélange de plusieurs saignées effectuées chez divers moutons hyperimmunisés. On trouvera leurs principaux caractères résumés dans le tableau 1.

2. Toxines employées.

Deux préparations de toxines diphtériques ont été employées : une toxine brute concentrée par ultra-filtration, et une toxine pure.

La toxine brute était préparée sur un milieu à base de viande (milieu Loiseau-Philippe). Les filtrats de culture ont été filtrés sur bougie et ensuite ultrafiltrés suivant le technique de van Ramshorst (1951). La toxine, une fois concentrée, a été dialysée contre du tampon phosphate K/K_2, pH $= 6{,}8 - 7{,}0$ M/15 + NaCl 0,172 M.

La toxine pure obtenue sous forme cristallisée titrait 3020 UF/mgN et donnait, testée par la technique de double diffusion en gélose contre un sérum complexe (sérum contenant un nombre élevé d'anticorps accessoires), une seule ligne de précipitation spécifique. La préparation ainsi que la purification de la toxine ont fait l'objet d'une autre publication (Relyveld *et al.*, 1958b, 1959b).

3. Précipitation spécifique en gélose.

Les techniques de précipitation spécifique selon Oakley et Fulthorpe (1953) et Ouchterlony (1949, 1950, 1953) telles que

TABLEAU 1.

Caractères du sérum de mouton No 5 brut et purifié.

	UF/ml	UI*/ml	UI*/UF	mgN/ml	UF/mgN	UI*/mgN	Am ** (μg)	% préc.	ym (μg)	1/ym (mg)	Am/UI* (μg)	UI*/Am (mg)
M5 non purifié	160	600	3,75	13,76	11,6	43,6	2065	6%	5,16	194	1,38	726
M5 purifié	752	3100	4,12	28,70	26,2	108	2713	17,8%	6,78	147,5	1,65	608

Am = N total anticorps précipitable correspondant à 400 unités UF (L_f) d'antitoxine.
ym = Am/400 = N anticorps précipitable contenu dans 1 unité UF d'antitoxine.
Am/UI = N anticorps précipitable contenu dans 1 unité internationale d'antitoxine.
1/ym = 400/Am = Nombre d'unités de floculation d'antitoxine par mg d'N précipitable.
UI/Am = Nombre d'unités internationales d'antitoxine par mg d'N précipitable.
* UI = Unités internationales (doses Lr).
** Valeurs corrigées d'après les expériences de radioactivité (voir tableaux 6 et 7).

nous les employons pour l'étude du système toxine-antitoxine ont été décrites en détail (Relyveld *et al.*, 1954, 1958b).

Les tubes employés avaient un diamètre de 8 mm. La première couche (0,5 ml) contenait du sérum à 100 UF/ml, gélosé à 0,75% + NaCl 1% + merth. 1:10.000. La couche intermédiaire (1 ml) contenait les mêmes concentrations de gélose, sel et merthiolate. La toxine non gélosée a été ajoutée à des concentrations différentes au-dessus de la couche intermédiaire.

La double diffusion sur plaque (Relyveld, 1958b) a été faite en employant de la gélose à 1,5% sur une épaisseur de 5 mm. Les concentrations de sel et merthiolate sont identiques à celles employées dans la technique en tubes.

4. Immuno-électrophorèse et électrophorèse en gélose.

Les immuno-électrophorèses, les électrophorèses en gélose, la coloration et la dessication des plaques ont été effectuées selon les techniques de Grabar et Williams (1953, 1955), Uriel et Grabar (1956), Williams (1954) et Williams et Grabar (1955), suivant des modalités décrites antérieurement (Relyveld *et al.*, 1956, 1958b).

Les mobilités électrophorétiques des constituants du sérum de mouton ont été comparées à celles des constituants du sérum de cheval et du sérum humain normal. Le sérum antisérum de cheval était un mélange de plusieurs saignées effectuées chez des boucs hyperimmunisées avec du sérum normal de cheval et certaines de ses fractions. Le mélange a été fait de façon à pouvoir détecter le plus grand nombre possible de constituants du sérum de cheval.

Les toxines employées pour la détection (U = Toxine brute concentrée, et T = Toxine pure) sont les mêmes que celles employées pour l'établissement des courbes de précipitation quantitative: ce sont également ces toxines qui ont été employées pour les expériences de précipitation spécifique en gel.

Les courbes d'extinction des bandes d'électrophorèse sur gélose ont été faites avec l'enregistreur d'extinction automatique de marque Zeiss (Oberkochen/Württ).

5. Etude quantitative de la réaction toxine-antitoxine.

Les courbes de précipitation ont été faites dans des conditions analogues à celles décrites par Pappenheimer et Robinson (1937).

Des quantités croissantes de toxine (brute ou pure) ont été ajoutées à 400 unités de floculation de sérum, et le volume final ajusté à 5 ml avec du tampon (PO_4KH_2/K_2H M/15 pH = 7,0 + NaCl 0,172 M). Après incubation à 37° pendant 2 heures puis séjour à 0° pendant 48 heures, les tubes ont été centrifugés pendant 10 minutes à 10.000 r.p.m. sur un centrifugeur de marque Sorvall, réfrigéré. Les précipités ont été lavés 2 fois par remise en suspension dans 5 ml de tampon, et ensuite dissous dans 1 ml de soude N/10. Le transfert dans des matras à minéralisation se fait à l'aide d'une pipette Pasteur; on lave le tube et la pipette deux fois avec 2 ml de la solution de soude. Les déterminations ont été faites en doubles.

Le titrage de l'azote a été fait après kjeldahlisation, l'ammoniaque a été distillé dans l'appareil de MARKHAM (1942), et titré au fur et à mesure de sa distillation par l'acide sulfurique N/10 ou N/70 (indicateur bleu de méthylène 0,05% + rouge de méthyle 0,1%) (VIGNAU, 1947).

Les déterminations suivantes ont été faites sur les surnageants:

a) Recherche de la toxine libre capable de réagir in vivo, par détermination de la dose minima réactive sur un lapin blanc.

La D.M.R. est la plus faible quantité de toxine qui, injectée sous un volume de 0,1 ml par voie intradermique, provoque au bout de 44 heures une zone d'érythème de 8 mm de diamètre (JENSEN, 1933). La concentration de toxine a été calculée d'après les diagrammes de JERNE (1951).

Nous avons divisé pour la représentation graphique le nombre de D.M.R. par 2.10^5 ce qui revient à admettre que: une unité de floculation de toxine contient 200.000 D.M.R. (RELYVELD et RAYNAUD, 1959a).

b) Recherche de la toxine libre capable de réagir in vitro par la technique de précipitation spécifique en gel (double diffusion en tubes).

La couche inférieure contenait 0,5 ml de sérum antidiphtérique gélosé (voir 3) à 20 UF/ml, la couche intermédiaire contenait 0,5 ml de gélose, le liquide surnageant (0,5 ml) a été versé sur la couche intermédiaire.

Deux types de sérums ont été employés: pour détecter la toxine, un sérum contenant presque uniquement l'antitoxine; dans ce cas nous avons employé le sérum de cheval 1451 dont la préparation a été décrite ailleurs; le sérum qui a servi à établir la courbe a été employé pour détecter la toxine ainsi que les autres antigènes capables de réagir avec les anticorps présents.

c) Recherche de l'antitoxine capable de neutraliser la toxine. Le titre neutralisant résiduel des surnageants a été déterminé par la technique classique de détermination du nombre d'unités internationales d'antitoxine (UI), sur cobayes, au niveau L + 1/1.

d) Recherche de l'antitoxine ainsi que des anticorps accessoires par la technique de double diffusion en gélose.

On a employé pour détecter l'antitoxine, une toxine pure, et pour détecter les anticorps accessoires, une toxine brute concentrée. La technique est identique à celle de (b).

e) Nous avons également déterminé la toxicité des précipités spécifiques. Le précipité lavé a été remis en suspension dans 5 ml de tampon et la toxicité déterminée par injection intradermique au lapin, comme pour les surnageants.

Les précipités spécifiques peuvent contenir un excès de toxine libre non neutralisée pour des valeurs du rapport toxine/antitoxine supérieures à celles du mélange L+; avec des sérums de faible pouvoir neutralisant, tous les précipités peuvent être toxiques. Une étude détaillée de ce phénomène a été faite avec des sérums de chevaux à Schick positif (Relyveld et Raynaud, 1959a).

6. Marquage de la toxine avec l'iode 131:

Nous avons mis au point une technique permettant de préparer un dérivé radioactif de la toxine sans que celle-ci subisse une baisse de la toxicité ou du pouvoir floculant [1]). La mise au point de cette technique a été décrite en détail (Relyveld, 1958b) et nous n'en donnons ici que les points principaux:

A 1 ml de solution d'iodure de sodium radioactif (activité 1 mc, pH = 8-9) contenant du bicarbonate de sodium à une concentration M/50, du carbonate et de l'hyposulfite de sodium à une concentration M/400, on ajoute dans l'ordre, les solutions suivantes:

Solution I : 1 ml
Solution II : 1 ml
H_2SO_4 N/70 : 4 ml
Solution III : 1 ml
Toxine : 10 ml
Tampon : 2 ml

Le mélange reste 1 heure à la température ordinaire, et est dialysé d'abord pendant une nuit contre de l'eau du robinet, et ensuite pendant 24 heures contre 5 litres de tampon phosphate. Le liquide extérieur est renouvelé 2 fois au cours des 24 heures.

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[1]) Masouredis (1957) a obtenu des résultats analogues en employant une technique d'ioduration légèrement différente.

Solution I.	Iode bisublimé :	32 mg
	Iodure de potassium :	167 mg
	Eau distillée q.s.p. :	100 ml
Solution II.	Iodate de potassium :	535 mg
	Eau distillée q.s.p. :	1 litre
Solution III.	Iodure de potassium :	2,32 g
	Eau distillée q.s.p. :	1 litre

La radioactivité a été déterminée sur des échantillons desséchés à l'étuve et correspondant à 0,1 ou 0,2 ml de liquide surnageant ou de la solution obtenue par redissolution du précipité. Nous avons utilisé un compteur Philips type PW 4035.

Les courbes de précipitation quantitative ont été faites avec la toxine radioactive dans les conditions décrites. Le précipité une fois lavé et dissous dans de la soude N/10, le volume est ensuite ajusté à 4 ml.

7. Digestion de la toxine:

La toxine a d'abord été dialysée contre du tampon phosphate de potassium 0,15 M + NaCl 0,172 M, pH = 7,61. La digestion a été faite avec de la trypsine cristallisée (Préparation commerciale de la firme Bios), activité 0,833. 10^{-3} $(\text{T.U.})^{\text{Cas}}$ par μg (Laskowski, 1955). L'inhibiteur de la trypsine préparé à partir du blanc d'oeuf selon la technique de Lineweaver et Murray (1947) a été employé pour arrêter la digestion.

A 4,5 ml de toxine dialysée contenant 6000 UF/ml, (Kf: $<1'$) ont été ajoutées 0,5 ml de solution de trypsine (2 mg dans 5 ml d'acide chlorhydrique 0,0025 N) et après digestion pendant 1 heure à 37°, 0,5 ml de solution d'inhibiteur (5 mg dans 5 ml de tampon). Titre après digestion: 700 UF/ml, (Kf : 17′).

Ces conditions ont été retenues à la suite de diverses expériences dont le détail a été rapporté ailleurs (Relyveld, 1958b).

Résultats.

La figure 1 montre les résultats des expériences de précipitation en gélose effectuées avec le sérum de mouton n° 5 purifié et une toxine pure. On remarque que, pour toutes les concentrations de toxine employées, il n'y a qu'une seule ligne de précipitation spécifique. Si la toxine pure (Fig. 2) est remplacée par une préparation brute, on

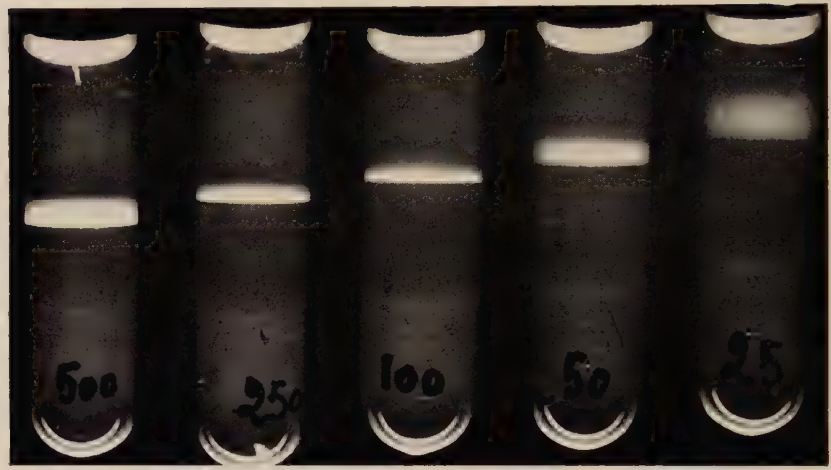

Fig. 1.
Précipitation spécifique en gel.
Sérum n° 5 purifié – Toxine pure (3020 UF/mg N).
Couche inférieure: sérum gélosé à une concentration de 100 UF/ml.
Couche intermédiaire: gélose sans sérum.
La toxine pure non gélosée a été ajoutée à une concentration de 500, 250, 100, 50 et 25 UF/ml (de gauche à droite) sur la couche intermédiaire.
La reproduction photographique représente seulement les couches inférieures.

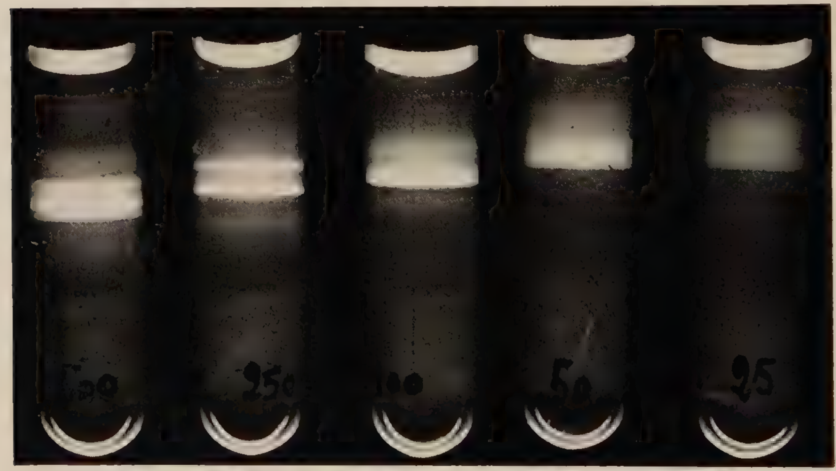

Fig. 2.
Précipitation spécifique en gel.
Sérum n° 5 purifié – Toxine brute ultrafiltrée (560 UF/mg N).
L'expérience a été faite dans les mêmes conditions que celle représentée sur la figure n° 1, mais la toxine pure a été remplacée par la toxine brute.

met aussi en évidence la réaction entre les constituants des systèmes accessoires. Les mêmes faits ont été observés avec le sérum n° 5 brut ainsi qu'avec d'autres préparations d'antitoxine du même type.

La vaccination préalable des animaux avec une anatoxine purifiée n'a pas empêché la montée des anticorps accessoires durant l'hyperimmunisation avec une anatoxine brute. Nous avons déjà montré que si on commence l'hyperimmunisation avec une anatoxine pure et si on remplace celle-ci durant l'hyperimmunisation par une préparation non purifiée, il y a formation d'anticorps accessoires à taux élevé (Raynaud *et al.*, 1959a), tandis que des traces d'impuretés présentes dans les préparations protéiques de haute pureté peuvent avoir le même effet (Finger et Kabat, 1958; Kabat, 1958; Raynaud *et al.*, 1959a; Relyveld *et al.*, 1957b).

La figure 3 montre les électroprotéinogrammes faits à partir des bandes d'électrophorèse en gélose après coloration et dessication. Les mobilités des constituants du sérum humain normal déterminées en électrophorèse libre dans les mêmes conditions de force ionique et de pH (Grabar, 1956; Williams et Grabar, 1955), permettent d'évaluer les mobilités des constituants des sérums étudiés.

La figure 4 montre les résultats de l'étude de la mobilité des anticorps par l'électrophorèse en gélose et par l'immuno-électrophorèse.

Pour comparer la mobilité électrophorétique des constituants sériques de mouton à celles de constituants plus connus, nous avons pris comme témoin le sérum de cheval, lequel a l'avantage de posséder des anticorps de mobilité différente. Les traits noirs qu'on a tracés sur la figure 4 correspondent à la position des sommets observés lors de l'électrophorèse du sérum normal de cheval (C.N.), effectuée dans les mêmes conditions expérimentales.

Le sérum n° 1451 est un sérum de cheval hyperimmunisé avec une toxine purifiée; on note la présence d'anticorps de mobilité beta-2. Le sérum de cheval antidiphtérique 441 contient des anticorps de mobilité beta-2 et gamma. Les deux sérums dont la préparation a été décrite ailleurs (Relyveld *et al.*, 1957b) présentent, à côté de l'antitoxine, peu d'anticorps accessoires, comme le montre la détection avec la toxine brute U et la toxine pure T.

Les mobilités électrophorétiques des constituants du sérum de mouton normal sont tout à fait différentes de celles du sérum normal de cheval. L'albumine (A) est le seul constituant ayant une mobilité à peu près égale, dans les 2 espèces animales.

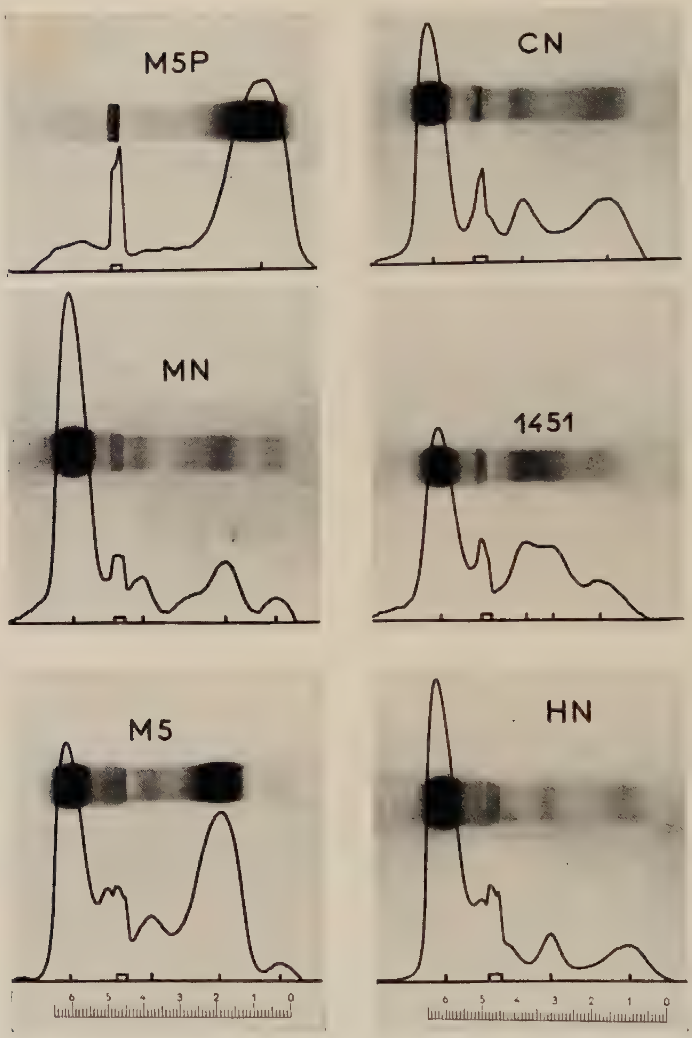

Fig. 3.

Electrophorèse en gélose des divers sérums, détermination des courbes d'extinction après coloration par l'Amidoschwarz.

H N = Sérum humain normal.
C N = Sérum de cheval normal.
1451 = Sérum de cheval antidiphtérique. No 1451 contenant des anticorps de mobilité beta-2 globulines.
M N = Sérum de mouton normal.
M 5 = Sérum de mouton antidiphtérique No 5.
M5P = Sérum de mouton No 5 purifié.

Dans les cupules de départ 0,15 ml de sérum dilué au 1/10, à l'exception du sérum M5P dilué au 1/20. Durée de l'électrophorèse 4 heures, tension 4 volts par cm.

L'échelle présente les mobilités des constituants du sérum humain normal déterminés en électrophorèse libre dans les mêmes conditions de pH et force ionique en $cm^2 . volt^{-1} . sec^{-1} \times 10^5$.

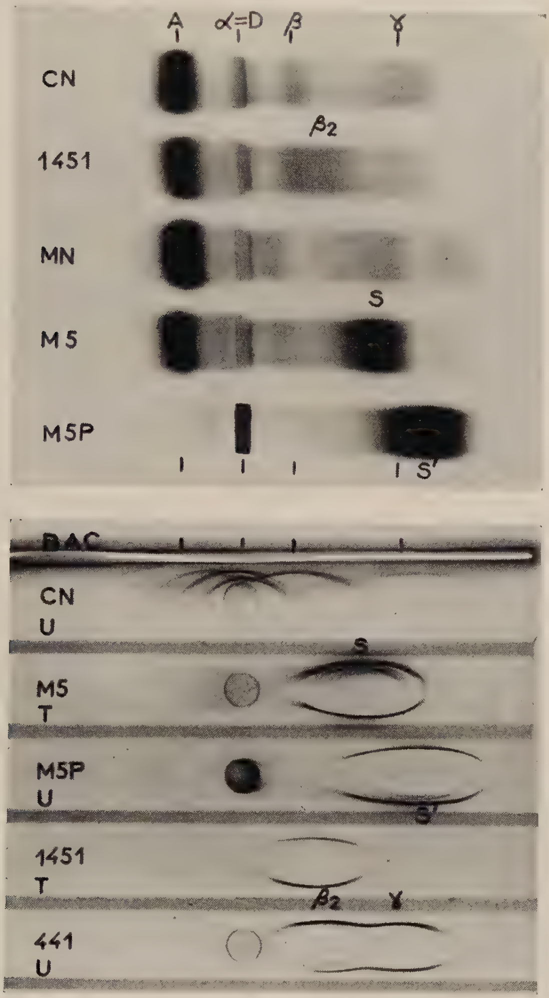

Fig. 4.

Immuno-électrophorèse et électrophorèse en gélose des sérums de mouton et de cheval.

C N = Sérum de cheval normal; 1451 = Sérum de cheval antidiphtérique contenant des anticorps beta-2 - globulines. 441 = Sérum de cheval antidiphtérique contenant des anticorps beta-2 et gamma-globulines.
M N = Sérum de mouton normal.
M 5 = Sérum de mouton antidiphtérique No 5.
M5P = Sérum de mouton No 5 après purification.
D = départ.
S = fraction sérique de mouton contenant les anticorps. S′ = anticorps de mouton après purification.
B.A.C. = Sérum de bouc antisérum de cheval. U = toxine diphtérique brute ultrafiltrée. T = toxine diphtérique pure. Dans les cupules de départ D:

1. – pour les électrophorèses en gélose, 0,15 ml de sérum dilué au 1/10 avec la gélose (excepté M5P 0,15 ml au 1/20).
2. – Immunoélectrophorèse. Le sérum est dilué avec la gélose à 100 UF d'antitoxine par ml; électrophorèse sur 0,1 ml. Le sérum de cheval normal (CN) a été dilué au 1/20. Dans les rigoles latérales 1,5 ml de toxine gélosée à 100 UF/ml et 1,5 ml de B.A.C. gélosé.
Durée de l'électrophorèse 4 heures à 4 volts par cm.

Nous remarquons un constituant ayant une mobilité intermédiaire entre celle des alpha et celle des beta-globulines de cheval, et un constituant peu concentré, ayant la mobilité des beta-2-globulines de cheval; dans la région des gamma-globulines, deux constituants, l'un plus rapide et l'autre moins rapide que les gamma-globulines de cheval.

L'électrophorèse du sérum hyperimmunisé montre l'augmentation du constituant "gamma" rapide, tandis que la concentration du constituant plus lent diminue. La concentration de l'albumine a également diminué, celle de la fraction de mobilité intermédiaire entre les alpha et les beta-globulines est augmentée. Nous voyons une fraction de mobilité alpha-1 plus détachée.

L'immuno-électrophorèse révèle que c'est la fraction gamma-globulines rapides (que nous proposons d'appeler gamma S [1]) qui contient la totalité des anticorps. Ces anticorps ont une mobilité intermédiaire entre celle des beta-2 et celle des gamma globulines de cheval.

La détection des anticorps avec une toxine brute ou pure permet de dépister les anticorps autres que l'antitoxine. Nous voyons que tous les anticorps ont leur maximum au même endroit mais que certaines des lignes de précipitation spécifique se prolongent jusque dans la région des alpha-globulines.

Nous avons voulu vérifier si les anticorps de mouton ont toujours la même mobilité électrophorétique ou si au contraire les anticorps sont au début de l'hyperimmunisation de mobilité différente (on sait que, chez le cheval, on observe une évolution de ce genre) (KECKWICK, 1941, KECKWICK et RECORD, 1941; RAYNAUD *et al.*, 1959a; RELYVELD *et al.*, 1957b et 1958b).

Les immuno-électrophorèses des saignées effectuées chez 6 moutons durant la première période de l'hyperimmunisation (titres in vitro compris entre 30 et 144 unités) n'ont pas montré de différence de mobilité électrophorétique des anticorps antitoxiques (tableau 1 bis).

De ce fait, on peut conclure que les anticorps antitoxiques précipitants de mouton sont toujours associés à la même fraction sérique.

Nous avons également fait l'analyse du sérum de mouton purifié: les anticorps ont une mobilité inférieure à celle des anticorps non digérés. Cette diminution de mobilité après digestion a déjà été

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[1]) Nous n'avons pas employé la lettre M signifiant déjà macroglobulines; S correspond à Sheep (anglais) ou Schaap (hollandais).

TABLEAU 1bis.

Caractères des sérums de mouton durant la première période de l'hyperimmunisation. Première injection le 13/10/58.

Sérum No	Dates de prélèvements le:	Titre		Lr/UF
		UF	Lr	
72	28/11/58	60	<145	<2,42
72	1/1/59	48	125	2,60
72	16/1/59	37	400	10,8
73	28/11/58	30	50	1,67
73	1/1/59	30	71	2,37
73	16/1/59	64	170	2,66
74	28/11/58	130	300	2,31
74	1/1/59	107	290	2,71
74	16/1/59	144	550	3,82
75	28/11/58	50	145	2,90
75	1/1/59	32	130	4,06
75	16/1/59	35	370	10,6
76	28/11/58	70	200	2,86
76	1/1/59	54	180	3,33
76	16/1/59	48	320	6,67
77	28/11/58	35	<80	<2,28
77	1/1/59	35	145	4,14
77	16/1/59	67	430	6.42

démontrée à plusieurs reprises (Relyveld, 1958b; van der Scheer *et al.*, 1941b; Williams, 1953).

Il n'y a, comme nous venons de le voir, formation que d'une seule ligne de précipitation en gélose entre la toxine pure et l'antisérum, cependant les anticorps dirigés contre cette molécule sont très hétérogènes. Diverses expériences faites avec la toxine partiellement digérée par la trypsine et les anticorps de cheval nous ont permis de mettre en évidence l'hétérogénéité de ces anticorps (Raynaud, 1959d; Relyveld, 1958b).

La figure 5 montre les résultats d'une expérience de double diffusion entre, d'une part la toxine pure non digérée (T) et la toxine digérée (TD) et d'autre part: 1) le sérum de mouton n° 5 (brut), 2) le sérum de cheval n° 825 (brut), 3) le sérum de cheval n° 906 (digéré), 4) le sérum de mouton n° 5 (digéré).

Nous avons antérieurement montré que le sérum 825, qui présente un rapport L+/Lf très bas et une faible avidité, ne donne avec la toxine digérée qu'une seule ligne nette correspondant à un anticorps précipitant et non neutralisant. Le sérum 906 donne avec la toxine digérée 2 lignes, l'une correspondant au système précédent

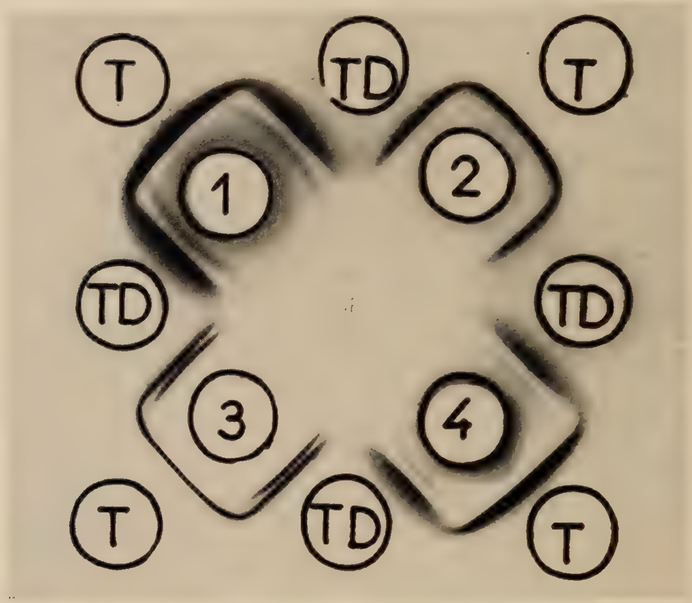

Fig. 5.

Mise en évidence des anticorps dirigés contre des groupes déterminants de la toxine par l'emploi d'une toxine digérée.

T = toxine pure non digérée: 0,2 ml à 200 UF/ml.
T.D. = toxine digérée: 0,2 ml à 200 UF/ml (titre déterminé après digestion).
1 – Sérum de mouton n° 5 non purifié à 160 UF/ml : 0.2 ml
2 – Sérum de cheval n° 825 dilué à 200 UF/ml : 0,2 ml
3 – Sérum de cheval n° 906 dilué à 200 UF/ml : 0,2 ml
4 – Sérum de mouton n° 5 purifié à 200 UF/ml : 0,2 ml
iamètre des réservoirs : 9 mm. Distance entre les bords des réservoirs: 7 mm.

(système précipitant non neutralisant), l'autre à un deuxième système qui pourrait être associé à l'antitoxine neutralisante [1]).

La toxine digérée donne avec les sérums de mouton au moins trois lignes de précipitation distinctes. Ces lignes donnent toutes la réaction d'identité avec celle de la toxine pure.

Une des lignes donne la réaction d'identité avec celle du système 825 – T.D.; la deuxième est identique à celle du deuxième système observable avec le sérum 906; la troisième ligne n'est visible que pour des concentrations élevées en T.D. et n'a pas été identifiée.

Le sérum de mouton antidiphtérique contient donc au moins 3 types d'anticorps dirigés contre des motifs antigéniques différents de la toxine.

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[1]) La nature non neutralisante du premier système (a_1–t_1) est bien établie sur le plan expérimental (Raynaud *et al.*, 1959c, d, Relyveld, 1958b). La nature du deuxiéme système observé avec la toxine digérée et tous les sérums de chevaux hyperimmunisés à beta-2-antitoxine (1451) ou à gamma antitoxine (441) n'est pas encore complètement élucidée.

Les tableaux 2 et 3 montrent les résultats de l'étude quantitative de la précipitation du sérum de mouton non purifié, avec d'une part la toxine brute et d'autre part la toxine pure. Les figures 6 et 7 montrent les courbes correspondantes.

TABLEAU 2.

Précipitation quantitative (sérum de mouton n° 5 non purifié - toxine brute).

UF ajoutée	mgN pté (Z)	In vivo			In vitro			
		Toxine non neutralisée *		Antitox non neutral	Anti-tox libre	Antic. libres	Toxi-ne libre	Antig libres
		Surnt	Pté	Surnt**	(1)	(2)	(3)	(4)
100	0,291	0	0	1380	1	2	0	0
200	1,02	0	0	—	1	2	0	0
400	2,16	0	0	1000	0	1	0	0
600	2,86	0	0	—	0	1	0	0
800	3,50	0	0	560	0	±	0	0
1000	4,17	0	0	480	0	±	0	±
1200	4,78	0	0	120	0	0	±	±
1600	5,02	0	0	0	0	0	1	2
2000	4,70	0,4	0,0007	0	0	0	1	2
2400	4,17	8	0,5	0	0	0	1	M
2800	3,79	110	1,35	0	0	0	1	M
3200	3,38	180	4,35	0	0	0	1	M

1 – Détermination avec une toxine pure.
2 – Détermination avec la toxine brute employée.
3 – Détermination avec un sérum de cheval ne contenant pas d'anticorps accessoires.
4 – Détermination avec le sérum employé.
Les chiffres indiquent le nombre de lignes de précipitation spécifique; M = lignes multiples. Antic. = anticorps; Antig. = antigènes; Surnt = Surnageant; Pté = Précipité.

* DMR/2.10^5; ** Unités internationales (doses L+).

L'azote total précipitable (Z) retrouvé avec ce sérum et une toxine brute dépasse largement les quantités retrouvées avec une toxine pure1). (Y=azote anticorps contenu dans le précipité, voir tableau 6).

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1) On rapporte également à titre de comparaison dans la figure représentant les résultats avec une toxine brute, l'azote précipité avec une toxine pure et vice versa.

TABLEAU 3.

Précipitation quantitative (sérum de mouton n° 5 non purifié – toxine pure).

UF ajoutée	mgN pté (Z)	In vivo			In vitro		
		Toxine non neutralisée *		Antitox non neutral	Antitox libre	Antic. libres	Toxine libre
		Surnt	Pté	Surnt**	(1)	(2)	(4)
100	0,233	0	0	—	1	a	0
200	0,932	0	0	1200	1	a	0
400	1,574	0	0	1000	0	a	0
600	1,95	0	0	—	0	a	0
800	2,24	0	0	560	0	a	0
1000	2,36	0	0	400	0	a	0
1200	2,31	0	0	240	0	a	1
1600	0,64	0	0,0007	0	0	a	1
2000	—	1,6	0,07	0	0	a	1
2400	—	80	<0,05	0	0	a	1
2800	—	237,5	—	0	0	a	1
3200	—	500	—	0	0	a	1

1 –
2 – } et abréviations voir tableau 2.
4 –

a : bandes très larges (au moins 2).

* DMR/2.10^5; ** Unités internationales (doses L+).

Le maximum de précipité avec la toxine brute se trouve même dans une région où déjà la plus grande partie du précipité spécifique toxine-antitoxine a été redissoute.

Les deux courbes montrent de façon irréfutable l'influence des systèmes accessoires. Pour l'étude quantitative d'un système antigène-anticorps déterminé, on doit donc s'assurer que le sérum ne contient pas d'anticorps qui peuvent réagir avec des antigènes accessoires, si on utilise un antigène brut, ou bien employer un antigène à l'état très pur.

Nous avons déjà montré dans l'étude des antisérums de cheval que la courbe obtenue avec une toxine brute et celle obtenue avec une toxine pure est la même si le sérum contient seulement des anticorps dirigés contre la toxine (Raynaud et Relyveld, 1959c; Relyveld, 1958b). Ce type de sérum donne par la technique de

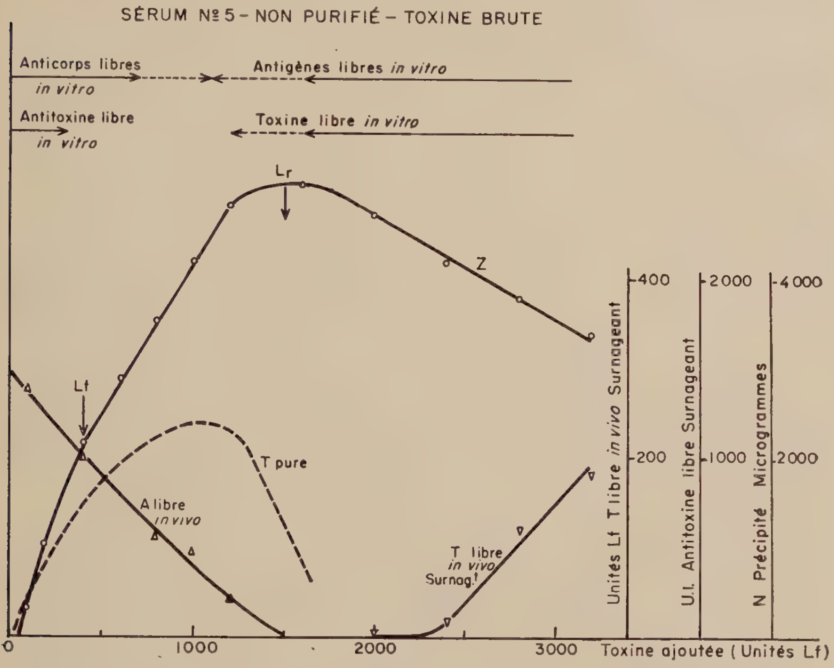

Fig. 6.

précipitation spécifique en gélose avec la toxine, à tous les stades de la purification, une seule ligne.

La forme de la courbe obtenue avec le sérum de mouton et la toxine pure n'est ni du type précipitine, ni du type floculation, mais est intermédiaire entre les deux.

Le précipité spécifique est partiellement soluble dans un excès d'anticorps ($T/A < 0{,}500$ en unités UF). Trois particularités permettent de reconnaître cette solubilité en excès d'anticorps :

1. La courbe ne passe pas rigoureusement par l'origine.
2. Le rapport Anticorps/Antigène calculé pour le précipité spécifique, présente un maximum pour $T/A = 0{,}500$ et baisse pour les valeurs inférieures (voir fig. 10).
3. On retrouve un pourcentage non négligeable (voir plus loin) de toxine marquée dans les surnageants pour $0 < T/A < 0{,}500$.

Le précipité devient ensuite complètement insoluble. Pour $T/A \geqslant 0{,}500$, on ne retrouve plus de toxine marquée dans les surnageants.

Le caractère intermédiaire entre le type précipitine et le type floculation est assuré par le fait que la zone d'équivalence in vitro très étendue ici aussi n'est pas rectiligne.

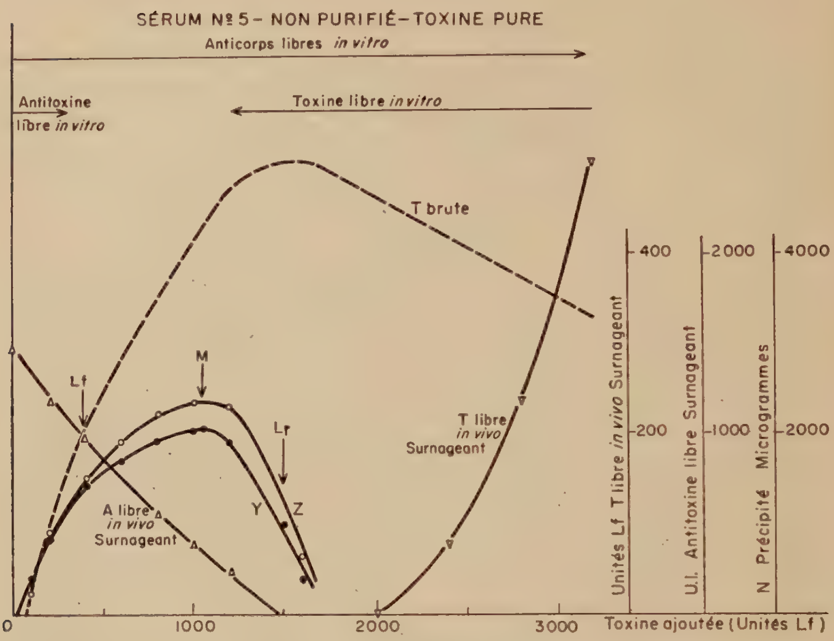

Fig. 7.

La solubilité des complexes antigènes anticorps en excès d'un des 2 réactifs a été attribuée, d'une part à la solubilité de l'antigène, et d'autre part à la solubilité des anticorps. La solubilité des complexes à beta-2-globulines en excès d'anticorps a été attribuée à la solubilité plus élevée de cette fraction globulinique (Grabar, 1958).

Nous avons déjà démontré que la mobilité électrophorétique des anticorps de mouton est intermédiaire entre la mobilité des beta-2 et celle des gamma-globulines de cheval. Leur point isoélectrique doit avoir aussi une valeur comprise entre celle du point isoélectrique des beta-2-globulines (5,6) et celle du point isoélectrique des gamma-globulines (7,6). (Deutsch et Nichol, 1948). Il est donc possible que dans les conditions de l'étude quantitative à pH = 7,0, les globulines de mouton soient plus solubles que les gamma-globulines de cheval et moins solubles que les beta-2-globulines de cheval. Ceci expliquerait l'allure intermédiaire de la courbe de précipitation.

Cohn et Pappenheimer (1949) ont étudié la réaction quantitative à pH = 5,2 et ont trouvé que la forme de la courbe des beta-2 globulines reste inchangée; ces auteurs considèrent en conséquence que la forme de la courbe n'a pas de rapport avec le point isoélectrique des fractions.

La courbe obtenue avec les anticorps de mouton est comparable à celle obtenue avec des beta-2-globulines de cheval, lorsque cette dernière est faite en milieu salin concentré (RAYNAUD et RELYVELD, 1959c; RELYVELD, 1958b). Dans ce dernier cas, la zone rectiligne d'équivalence persiste, mais les précipités sont peu solubles en excès d'anticorps. Il reste cependant une différence importante entre les deux types de courbes; avec le sérum de cheval, l'azote anticorps dans le précipité n'augmente pas de façon nettement appréciable tout le long de la zone d'équivalence. La figure 7 montre qu'il n'en est pas ainsi dans le cas du sérum de mouton: on observe au contraire entre les point Lf et M, une forte augmentation de l'N anticorps précipité.

La position du point Lr sur la courbe pose divers problèmes qu'il convient d'examiner en détail.

Avec une toxine brute, ce point se trouve juste au maximum de la courbe et donne l'impression erronée, que le maximum de précipitation est en rapport avec l'équivalence de la réaction toxine-antitoxine. La courbe faite avec une toxine pure montre que le point Lr se trouve en réalité bien après le maximum de précipitation, et même dans la zone descendante.

L'étude des courbes de précipitation quantitative avec des sérums de chevaux sans immunité de base (sérums ayant un pouvoir précipitant élevé et un pouvoir neutralisant bas) a montré que le point Lr peut se trouver dans la portion ascendante de la courbe et qu'on peut même avoir présence de toxine non neutralisée dans toute la zone de précipitation. Nous avons montré par des expériences de neutralisation et d'épuisement que ces sérums contenaient peu d'anticorps neutralisants par rapport aux anticorps précipitants.

Les sérums de mouton étudiés contenant un taux d'anticorps neutralisants plus élevé que le taux d'anticorps précipitants, présentent la disposition inverse.

La courbe montre aussi de nouveau, comme nous l'avons déjà signalé pour les sérums de chevaux, que le point Lf ne concorde pas toujours avec le point Lr; ce dernier, suivant la concentration en anticorps neutralisants peut se trouver à gauche ou à droite du point Lf.

Le dépistage des anticorps in vitro par la technique de diffusion en gélose met de nouveau en évidence le rôle des systèmes accessoires. Avec une toxine pure, on précipite seulement les anticorps

TABLEAU 4.

Précipitation quantitative (sérum de mouton n° 5 purifié – toxine pure).

UF ajoutée	mgN pté (Z)	In vivo			In vitro		
		Toxine non neutralisée *		Antitox non neutral.	Antitox libre	Antic. libres	Toxine libre
		Surnt	Pté	Surnt**	(1)	(2)	(4)
100	0,495	0	0	—	1	2	0
200	1,05	0	0	1125	1	2	0
400	1,835	0	0	875	0	a	0
600	2,32	0	0	—	0	a	0
800	2,56	0	0	687,5	0	a	0
1000	2,76	0	0	562,5	0	a	0
1200	3,01	0	0	337,5	0	a	±
1600	3,14	0	0	107,5	0	a	1
2000	2,66	0,012	0,001	0	0	a	1
2400	1,906	3,5	0,5	0	0	a	1
2800	1,37	67,5	2,5	0	0	a	1
3200	0,84	145	2,2	0	0	a	1

1 –
2 – } et abréviations voir tableau 2.
4 –

a : une ou plusiers bandes très larges.
* DMR/2.10^5; ** Unités internationales (doses L+).

antitoxiques ; les anticorps accessoires restent dans les surnageants et peuvent être mis en évidence avec une toxine brute.

Les tests de diffusion montrent également qu'il n'y a plus d'antitoxine libre détectable in vitro après le point 200 ; on retrouve par contre dans les surnageants de la toxine libre in vitro avant le point Lr. Nous examinerons ces faits en détail lors de l'étude quantitative avec une toxine radioactive.

La neutralisation de la toxine par l'antitoxine se fait de la même façon (dans le cas de la toxine brute et de la toxine pure). Les courbes donnant le taux d'anticorps neutralisants des surnageants sont identiques.

On note une différence dans les quantités de toxine libre dans les surnageants ; la présence dans la toxine brute de dérivés atoxiques ou partiellement dénaturés peut en être la cause, car les taux de

TABLEAU 5.

Précipitation quantitative (sérum de mouton n° 5 purifié – toxine brute).

UF ajoutée	mgN pté (Z)	In vivo			In vitro			
		Toxine non neutralisée *		Antitox non neutral.	Anti-tox libre	Antic. libres	Toxi-ne libre	Antig. libres
		Surnt	Pté	Surnt**	(1)	(2)	(3)	(4)
100	0,379	0	0	—	1	2	0	0
200	1,08	0	0	1125	1	2	0	0
400	2,30	0	0	875	0	1	0	0
600	3,28	0	0	—	0	±	0	0
800	4,05	0	0	687,5	0	±	0	0
1000	4,59	0	0	500	0	0	0	0
1200	5,01	0	0	375	0	0	±	±
1600	5,98	0	0	140	0	0	1	2
2000	6,42	<0,0025	0,00025	0	0	0	1	2
2400	6,66	0,9	<0,02	0	0	0	1	3
3000	6,44	70,5	2,5	0	0	0	1	3
3600	6,16	455	6,75	0	0	0	1	3
4200	6,05	940	14,5	0	0	0	1	5
4800	6,04	2610	3,5	0	0	0	1	M

1 –
2 –
3 –
4 –
} et abréviations voir tableau 2.

* DMR/2.10^5; ** Unités internationales (doses L+).

toxine libre active in vivo sont plus faibles pour cette dernière que pour la toxine pure.

Les précipités obtenus après le point Lr sont seulement faiblement toxiques. Le même fait a été observé avec les gamma-globulines de cheval (gamma-globulines ayant un rapport L+/Lf élevé), tandis que les précipités spécifiques obtenus avec les globulines beta-2 de cheval ainsi qu'avec les antitoxines de chevaux à Schick positif (bêta-2 et gamma-globulines), peuvent contenir des quantités élevées de toxine non neutralisée.

Les tableaux 4 et 5, ainsi que les figures 8 et 9, montrent les résultats obtenus avec le sérum de mouton purifié.

On note de nouveau l'influence des anticorps accessoires: avec la

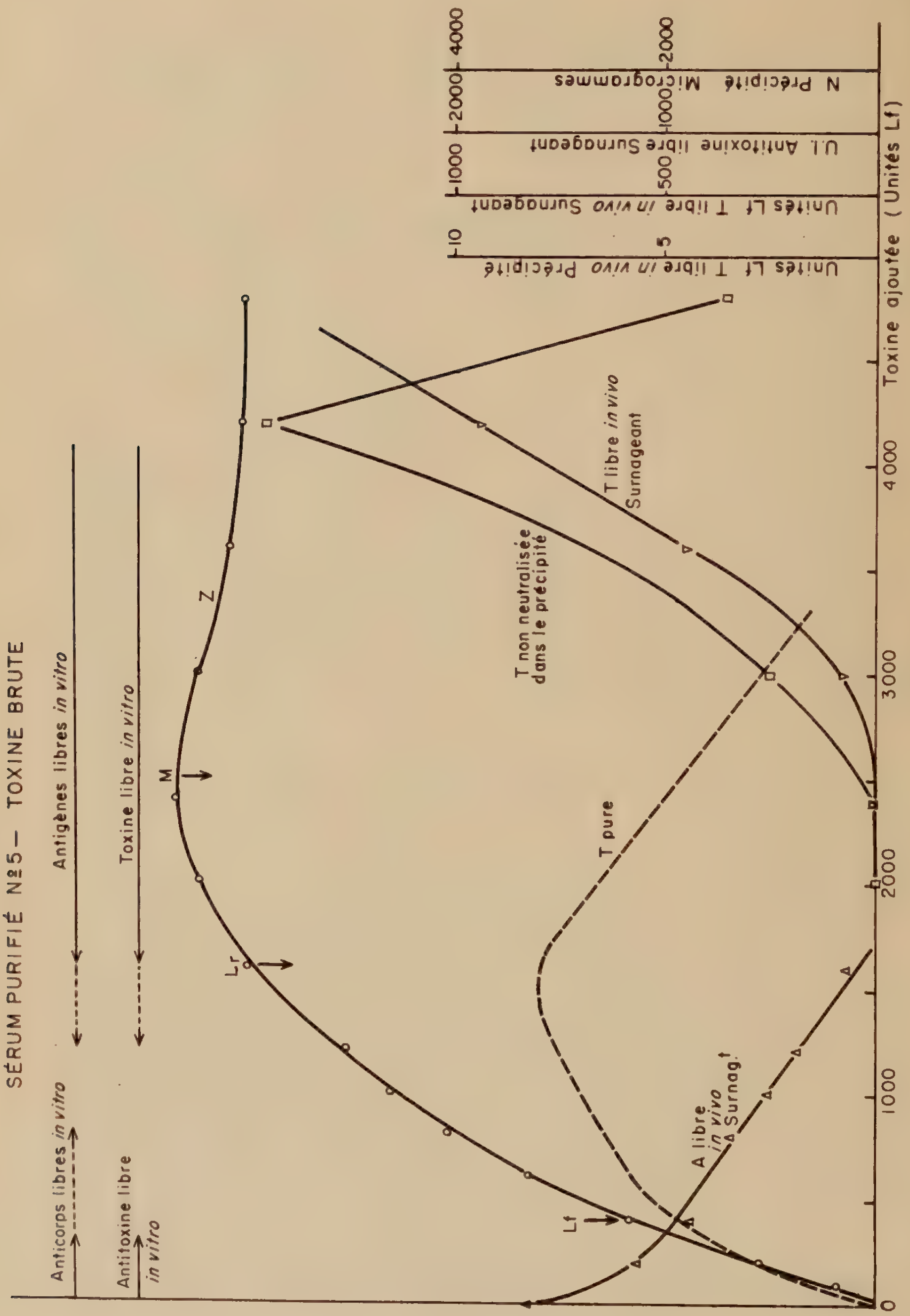
SÉRUM PURIFIÉ N° 5 — TOXINE BRUTE
Anticorps libres *in vitro*
Antigènes libres *in vitro*
Antitoxine libre *in vitro*
Toxine libre *in vitro*
Lf
Lr
M
Z
T non neutralisée dans le précipité
T libre *in vivo* Surnageant
T pure
A libre *in vivo* Surnag.t
Unités Lf T libre *in vivo* Précipité
Unités Lf T libre *in vivo* Surnageant
U.L. Antitoxine libre Surnageant
N Précipité Microgrammes
10
5
1000
500
2000
1000
4000
2000
0
1000
2000
3000
4000
Toxine ajoutée (Unités Lf)

Fig. 8.

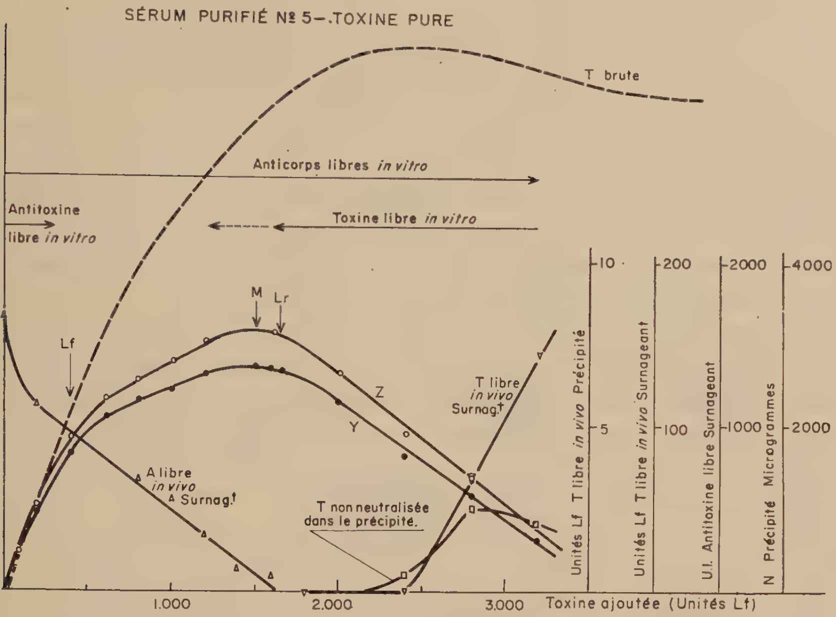

Fig. 9.

toxine brute, il y a beaucoup plus de précipité et la courbe devient très étendue. La zone d'équivalence in vitro est plus prononcée et plus longue qu'avec le sérum non purifié, le même fait a été observé avec les sérums de chevaux digérés. La dissolution du précipité spécifique dans un excès de toxine se fait moins vite, les précipités deviennent toxiques dès que l'on a atteint le point Lr.

Les anticorps digérés semblent d'après ces données encore moins solubles que les anticorps natifs. Nous avons déjà démontré que leur mobilité électrophorétique est moins élevée. Le point Lr se trouve dans ce cas juste après le maximum de précipitation.

Les observations relatives à la comparaison des titrages in vitro et in vivo, dans les surnageants, faites pour les sérums non purifiés, sont valables ici aussi.

L'emploi d'une toxine radioactive nous a permis de rechercher la présence d'anticorps non précipitants et de calculer la composition moléculaire des complexes toxine-antitoxine tout au long de la courbe de précipitation quantitative.

La toxine marquée employée titrait 3020 UF/mg N et nous avons introduit un facteur de correction basé sur la valeur 3100 UF/mgN pour une toxine pure; 97,5% de la radioactivité doivent être précipitables par l'antitoxine.

La quantité de toxine contenue dans le précipité spécifique a été calculée d'après la radioactivité du précipité et du surnageant.

La radioactivité totale retrouvée nous a renseignés sur la validité de la technique employée et sur les pertes survenues pendant les lavages.

On doit tenir compte du fait que cette méthode introduit plusieurs sources d'erreurs et que les résultats ne peuvent être considérés comme rigoureusement exacts. Elle permet néanmoins d'obtenir dans le but que nous nous sommes proposés, des approximations très valables.

La figure 10 représente pour le sérum non purifié, l'azote total précipité (Z), la radioactivité ajoutée[1]), le nombre d'i.p.m retrouvés dans le surnageant, le nombre d'i.p.m retrouvés dans le précipité et la composition moléculaire des précipités spécifiques.

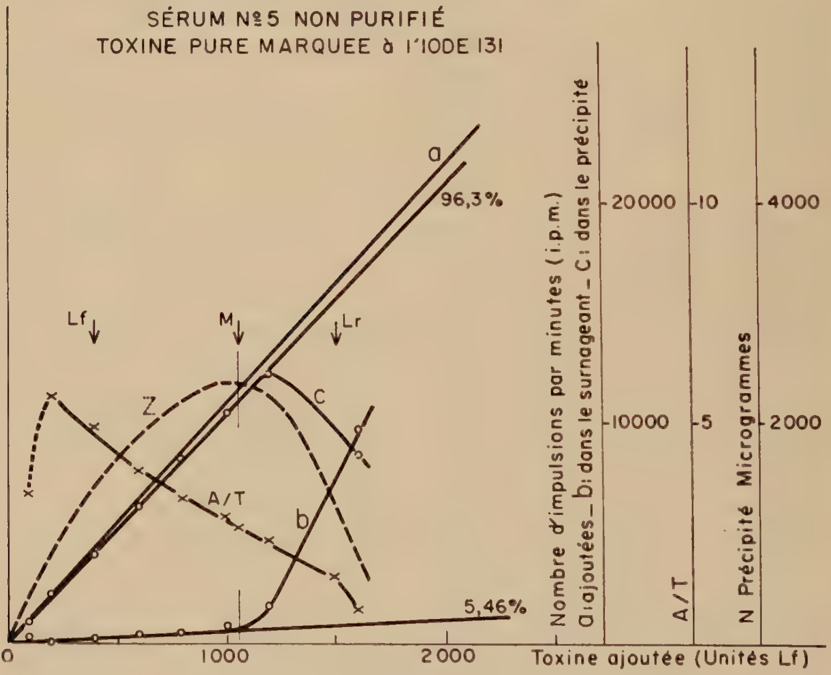

Fig. 10.

Le pourcentage de toxine précipitée, pour toute la première partie de la courbe jusqu'au maximum, varie peu. On retrouve en moyenne 96,3% de la toxine dans le précipité et 5,46% dans le surnageant ¹). Ces données permettent de calculer pour une toxine pure, 3140 UF/mgN d'après la radioactivité retrouvée dans les précipités et 3163 UF/mgN d'après la radioactivité retrouvée dans les surnageants. Ces valeurs sont proches de celles que nous avons admises pour la toxine pure: 3100 UF/mgN.

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[1]) Nombre d'impulsions par minute (i.p.m), correspondant aux unités de floculation de la toxine.

On peut donc considérer que pour la partie ascendante de la courbe, comprise entre les valeurs 200 et 1060 UF, toute la toxine ajoutée se trouve dans le précipité spécifique. Après le maximum de la courbe, on retrouve de la toxine dans le surnageant en quantité de plus en plus élevée.

Tout à fait au début de la courbe, on retrouve cependant une certaine proportion de toxine marquée dans le surnageant, le précipité spécifique étant un peu soluble dans un grand excès d'anticorps.

La détermination du poids moléculaire des globulines de mouton a été faite sur la fraction sérique contenant les anticorps obtenus par précipitation saline. La constante de sédimentation S_{20} est de $6,6 \times 10^{-13}$ sec et la constante de diffusion D_{20} est de $3,8 \times 10^{-7}$ cm²/sec. Le poids moléculaire correspondant est de 175.000 (± 10%). La présence d'une trace d'un composant plus lourd dans la préparation a fait supposer que le poids moléculaire de la fraction principale est en réalité un peu moins élevé et peut être évalué à 170.000 ²).

Le tableau 6 présente la composition moléculaire des précipités calculée en utilisant cette valeur. Les calculs ont été faits d'après la moyenne des quantités de toxine retrouvée dans les surnageants et dans les précipités.

La floculation initiale se fait pour une composition moléculaire $A/T = 4,9$, qu'on peut assimiler à A_5T ou $A_{10}T_2$. Le précipité a au maximum de la courbe une composition $A_{2,6}T$ ou proche de A_5T_2.

Le complexe correspondant au maximum de précipitation contient donc une proportion d'anticorps, par rapport à l'antigène, beaucoup plus faible que le précipité donnant la floculation la plus rapide (floculation initiale).

La constante de sédimentation du sérum digéré S_{20} est égale à

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¹) La fraction de 5,46%, présente en permanence dans les surnageants correspond aux impuretés autres que la toxine, présentes dans la préparation utilisée.

²) Les déterminations de poids moléculaires ont été effectuées par le Docteur P. F. Mynlieff, laboratoire van 't Hoff (Utrecht).

TABLEAU 6.

Détermination de la quantité de toxine dans le surnageant et dans le précipité. Composition moléculaire antitoxine/toxine. (Sérum de mouton n° 5 non purifié – Toxine pure marquée à l'iode 131).

Toxine marquée			Précipité			Surnageant				% moyen	μgN tox. ds le pté (x)	μgN total ds le pté (z)	μgN antit. ds le pté (y)	y/x	A/T*
UF	μgN	ipm	ipm	%	% corrigé	ipm	%	100 % –% S	% corrigé						
100	32	1094	950	87	89,1	297	27,2	72,8	74,6	81,9	26	233	207	8	3,4
200	65	2189	2300	105	107,7	60	2,72	97,28	99,8	103,8	65	932	867	13,3	5,6
400	129	4378	4120	94	96,4	166	3,79	96,21	98,7	97,6	126	1574	1448	11,5	4,9
600	193	6568	6248	95,2	97,6	304	4,63	95,37	97,9	97,8	189	1950	1761	9,3	3,9
800	258	8757	8560	97,7	100,2	541	6,18	93,82	96,2	98,2	253	2240	1987	7,9	3,3
1000	322	10946	10600	96,8	99,3	842	7,70	92,30	94,7	97	312	2360	2048	6,6	2,8
1060	342	Max		97,7	100,2		5,82	94,18	96,6	98	335	2400	2065	6,2	2,6
1200	387	13135	12360	94,1	96,6	1735	13,2	86,8	89	92,8	359	2310	1951	5,4	2,3
1500	483	Lr		57,8	59,2		45,8	54,2	55,6	57,4	277	1280	1003	3,6	1,5
1600	515	17514	8640	49,3	50,6	9882	55,7	44,3	45,5	48,1	248	640	392	1,6	0,67

* A/T = Antitoxine/toxine (Composition moléculaire) = y/x × 72.000/170.000 = y/x × 0,4235.
Les valeurs des points Max et Lr ont été calculées d'après les courbes de radioactivité.

5×10^{-13} sec; la constante de diffusion D_{20} est de $5{,}5 \times 10^{-7}$ cm^2/sec. Le poids moléculaire correspondant est de 93.000 (± 10%).

La présence dans la préparation d'un composant de poids moléculaire plus faible, fait que le poids moléculaire de la fraction principale est plus élevé. Nous avons adopté la valeur de 100.000 1)).

La figure 11 et le tableau 7 montrent les résultats de l'étude de la réaction entre le sérum purifié et la toxine radioactive. On retrouve de nouveau pour la première partie de la courbe, une quantité faible de radioactivité dans le surnageant, en moyenne 4,6 % de la radioactivité ajoutée.

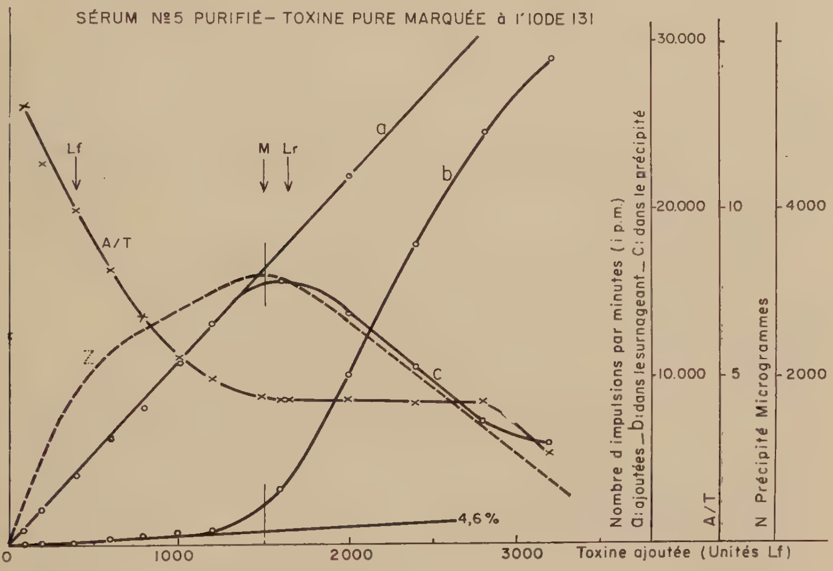

Fig. 11.

Les résultats sont un peu moins réguliers pour le précipité, mais montrent de nouveau que la presque totalité de la toxine se retrouve dans le précipité, sauf en zone de grand excès d'anticorps où la combinaison spécifique est partiellement soluble.

La floculation initiale se fait pour une composition moléculaire $A_{9,9}T$ et le précipité a au maximum de la courbe, une composition égale à $A_{4,4}T$. Ces valeurs sont proches des valeurs entières $A_{20}T_2$ et A_9T_2. La floculation initiale se fait donc, comparée à celle qui s'observe avec le sérum non purifié, avec deux fois plus de molécules

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1) voir note 2) page 395.

TABLEAU 7.

Détermination de la quantité de toxine dans le surnageant et dans le précipité. Composition moléculaire antitoxine/toxine.
(Sérum de mouton n° 5 purifié - Toxine pure marquée à l'iode 131).

Toxine marquée			Précipité			Surnageant				% moyen	µgN tox. ds le pté (x)	µgN total ds le pté (z)	µgN antit. ds le pté (y)	y/x	A/T*
UF	µgN	ipm	ipm	%	% cor-rigé	ipm	%	100 % −%S	% cor-rigé						
100	32	1094	830	76	78	164	15	85	87,2	82,6	26	495	469	18,0	13,0
200	65	2189	2030	92,7	95	117	5,36	94,64	97	96	63	1050	987	15,7	11,3
400	129	4378	4080	93,3	95,8	176	4,02	95,98	98,6	97,2	125	1835	1710	13,7	9,9
600	193	6568	6300	96	98,4	229	3,49	96,51	99	98,7	190	2320	2130	11,2	8,1
800	258	8757	8028	91,6	94	524	5,97	94,03	96,4	95,2	245	2560	2315	9,5	6,8
1000	322	10946	10800	98,6	101	782	7,2	92,8	95,2	98,1	316	2760	2444	7,7	5,6
1200	387	13135	13100	99,8	102	828	6,3	93,7	96	99	383	3010	2627	6,9	4,9
1500	483	Max		94	96,4		13,4	86,6	88,9	92,7	447	3160	2713	6,1	4,4
1600	515	17514	15600	89	91,2	3312	18,8	81,2	83,4	87,3	449	3140	2691	6	4,3
1650	531	Lr		88,7	91		21,1	78,9	80,8	85	451	3120	2669	5,9	4,3
2000	644	21892	13760	62,8	64,4	10080	46	54	55,4	59,9	386	2660	2274	5,9	4,3
2400	773	26270	10500	40	41	17840	68	32	32,8	36,9	285	1906	1621	5,7	4,1
2800	902	30649	7120	23,2	23,8	24480	79,8	20,2	20,7	22,3	201	1370	1169	5,8	4.2
3200	1031	35027	5960	17	17,45	29000	82,8	17,2	17,7	17,6	181	840	659	3,6	2,6

*A/T = Antitoxine/toxine (Composition moléculaire) = y/x × 72.000/100.000 = y/x × 0,72.

Les valeurs des points Max et Lr ont été calculées d'après les courbes de radioactivité, d'anticorps. Au maximum de la courbe, le rapport moléculaire antitoxine/toxine est approximativement la moitié de la valeur observée pour le mélange Lf.

La composition moléculaire des précipités formés dans toute la partie descendante de la courbe est la même, voisine de A_4T.

Le dernier précipité a une composition moléculaire antitoxine toxine bien définie et les complexes contenant plus de toxine sont solubles.

DISCUSSION.

Les sérums de mouton étudiés présentent une particularité remarquable: le titre des anticorps neutralisants y est beaucoup plus élevé que celui des anticorps floculants. Leur rapport Lr/Lf est élevé (3,75 pour le sérum brut). Cette particularité persiste après digestion pepsique en s'accentuant (rapport Lr/Lf = 4,12). Il a été demontré (VAN TRIET, 1959) que la dissociation entre les titres retrouvés "in vivo" et "in vitro" sont indépendants du niveau du titrage "in vivo"; les valeurs trouvées au niveau: Lr/10, Lr/100, Lr/1000 sont les mêmes.

Cette dissociation des pouvoirs neutralisant et floculant est inverse de celle que l'on observe dans le cas des sérums de chevaux Schick positifs. Son étude nous a donc paru particulièrement intéressante. On peut concevoir à priori diverses hypothèses pour expliquer les variations considérables que l'on peut ainsi observer suivant ces cas dans l'évolution des 2 pouvoirs floculant et neutralisant.

Dans le cas qui nous occupe où le pouvoir neutralisant est plus élevé que le pouvoir floculant, on peut a priori admettre que ces anticorps neutralisants sont soit des anticorps non précipitants, soit des anticorps coprécipitants. Les anticorps non précipitants sont faciles à mettre en évidence par l'emploi d'un antigène marqué (RELYVELD, 1958b; BURTIN, 1955). Les résultats rapportés plus haut indiquent nettement que les anticorps neutralisants ne sont pas de ce type, mais de type coprécipitant analogues à ceux qui ont été mis en évidence dès 1935 par HEIDELBERGER et KENDALL.

Nous nous proposons de revenir ultérieurement sur ce point particulier pour préciser la nature de ce type d'anticorps.

Résumé.

Nous avons étudié les sérums de mouton antidiphtériques préparés par l'un d'entre nous (VAN TRIET, 1959). Ces sérums présentent un pouvoir neutralisant élevé et un pouvoir floculant modéré (rapports Lr/Lf de l'ordre de 3 à 4).

Nous avons montré que les anticorps précipitants antidiphtériques sont associés pendant toute la durée de l'hyperimmunisation à des globulines de mobilité intermédiaire entre celle des beta-2 et celle des gamma globulines de cheval. Nous proposons d'appeler ces globulines anticorps de mouton globulines gamma-S.

Ces sérums contiennent de nombreux anticorps accessoires, mais l'emploi d'une toxine diphtérique de haute pureté a permis d'établir la courbe de précipitation correspondant au système antitoxine-toxine. Cette courbe présente une allure intermédiaire entre le type précipitine et le type floculation. Le précipité spécifique est partiellement soluble dans un excès d'anticorps, la courbe a une forme arrondie dans le cas du sérum brut; le sérum purifié présente une zone rectiligne. La zone d'équivalence in vitro est dans les deux cas très étendue; dans la région comprise entre les points Lf et M, on ne retrouve par précipitation en gel ni antitoxine ni toxine dans les surnageants. Ces derniers contiennent cependant des taux élevés d'antitoxine neutralisante mesurables par les tests in vivo. Ces taux d'antitoxine neutralisante diminuent progressivement entre les points Lf et Lr. Le point M se situe avant le point Lr.

L'emploi d'une toxine marquée à l'iode 131 nous a montré qu' entre les points Lf et M, l'antigène était complètement précipité et ne donnait pas de complexes solubles avec les anticorps neutralisants présents dans les surnageants. Ces anticorps neutralisants sont coprécipités lorsqu'on augmente le rapport antigène/anticorps.

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COMPARISON OF THE FELIX AND CALLOW SYSTEM OF PHAGE TYPING OF *SALMONELLA PARATYPHI* B WITH THE NATURAL SYSTEM USED IN THE NETHERLANDS

RESULTS OF MEMBERS OF THE FIRST SUBCOMMITTEE ON *PARATYPHI* B OF I.C.E.P.T.

by

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INTRODUCTION.

The question of phage typing of *Salmonella paratyphi* B has two aspects, one theoretical and one practical. Now some people have a poor opinion of theoretical scientific discussions. Such discussions begin where matters stop to have a practical significance. Indeed, there exists a kind of theoretical speculation that has given rise to this opinion; however, an attempt to see similarities and relationships has always been at the beginning of progress and, therefore, the best proof of a theory is the practical result. It is generally agreed that in the study of a varietal subdivision of a bacterial species by means of bacteriophages two phenomena play a role; first the adaptations, and secondly lysogenicity. In the case of *S. typhi* the adaptations are of primary importance; however the study of lysogenicity, started by ANDERSON and FELIX (1953) and worked out further by ANDERSON and his co-workers, has given an insight in the relationship of types, and the structure of their resistances. The importance given to these matters is such, that new types are considered only incompletely described, if the natural phages have been overlooked.

The view that types are not only determined by adaptations, but also by lysogenicity, is accepted by NICOLLE and co-workers (1951) and ANDERSON (1956). These lines are, however, not followed up in the extension of the typing scheme suggested in the paper of ANDERSON. The concept, that types are determined by adaptation as well as by lysogenicity is followed up in the natural system for *S. paratyphi* B. This, however, is not a system, wherein types are, as a routine, diagnosed by the demonstration of the natural phages.

The pattern of phage resistances and sensitivities of *S. paratyphi* B differs from that of *S. typhi*, only a few adaptations of phage 1 of FELIX and CALLOW are found. Thus lysogenicity comes forward as an instrument for phage typing. By means of the study of lysogenicity a natural typing system could be drawn up, wherein the strains of all types, such as it is seen in *S. typhi*, possess similar lysogenic properties.

In 1955 a Subcommittee was appointed by Dr FELIX to compare the practical results obtained with the FELIX and CALLOW system with those obtained with the natural system. As a result of this rejoined effort it was found that in many cases a correction of the diagnoses obtained by means of the FELIX and CALLOW system was necessary.

I express my thanks to Dr BEUMER, Professor BRANDIS and Dr RISCHE, who helped to perform this work, and to the members of the First Subcommittee on *Salmonella paratyphi* B who confined themselves to a confirmation of the reactions of the phages and strains sent to them.

TECHNIQUE.

1. Phage reactions. The technique indicated by CRAIGIE and FELIX (1947) and FELIX and CALLOW (1951) was followed.

2. Demonstration of the natural phages. A survey of the phages present in the strains may be obtained as follows: The strain to be examined as to its natural phage was grown in mixed culture with strain 3aB62 for 24 hours. In this mixed culture the type determinating phages I, IV and VI are found. Moreover, a group determinating phage with lytic spectrum d, serology II was found in strain B 2553, the type strain of type 3b variation I of Dr FELIX, and in some strains of type Midwoud[1]), all isolated from the same epidemic (in filtrates of fresh broth cultures of these strains these phages are always found). It may be remarked here, that in the strains of type Midwoud described before (SCHOLTENS, 1950,

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[1]) Types Beccles-Midwoud and Beccles-22 were described as "Midwoud" and "22" before (SCHOLTENS, 1956).

1952, 1956) phages with lytic spectrum d were found only after a longer incubation with strains or with filtrates of strains of different types. The preparation obtained is tested on the strains indicated in table 1. The aspect of the plaques or of the confluent lysis is a help for the diagnosis.

TABLE 1.

Routine demonstration of the natural phages of *S. paratyphi B.*

Test strains		Reactions of the test strains to the natural phages				
Strain	Type	I	IVb	IVa	VI	d
14502	2	–	–	cl	cl	cl
1153	Jersey	–	cl	cl	cl	cl
821	Beccles-Midwoud	–	cl	cl	cl	–
B62	3a-common type	cl	cl	cl	cl	cl
818	"18"	cl	–	–	–	cl
B2500	3a variation 3	cl	–	–	–	cl
1131	Taunton-Kampen	–	–	cl	–	cl

Reactions to directly demonstrable natural phages of the strains utilised in the examination for the presence of these phages in strains of *S. paratyphi B.*

A serological confirmation of the identity of the phages found can be obtained by growing the strain with strain 3aB62 in broth to which specific immune serum is added for 24 hours, as described in an earlier paper (SCHOLTENS, 1955). The preparation obtained is tested on the strain indicated in table 1. This confirmation should be obtained if a diagnosis is to rely on the demonstration of the natural phage. For scientific purposes, however, the phages should be titrated and examined in the critical test concentration with serial serum dilutions.

3. The medium. Mince 0.5 kg meat and incubate for 24 hours with 1 litre water at 4° C. Boil for 20 minutes and filter through cloth. Bring the volume up to 1 l. Add 12.5 g Difco pepton, 7.5 g NaCl and 20 g agar powder. Heat for 20 minutes at 115° C. and adjust the pH to 7.3 with a solution of 15% Na_2CO_3. Sterilise at 115° C.

The medium differs from the medium described earlier (SCHOLTENS, 1950) only in the adjustment of its pH to 7.3.

Larger volumes of the medium may be prepared and stored for a few months.

4. The choice of the typing phages. Two serological varieties of the phages with the lytic spectra c, d, e and h have been found, namely phages with the serology II and phages with the serology VI. The phage reactions with the preparations with serology VI are more conspicuous than those obtained with the phages with the serology II. On the other hand, the preparations with the serology VI are less stable. Especially the phages c, VI (lytic spectrum c, serology VI) examined were unstable. Therefore, and also because all phages of this series can be obtained, the phages of the serology II were chosen. The phages sent to the members of the first Subcommittee were the phages c, II; e, II; f, II and h, VI, and the adaptation of phage 1 of Felix and Callow to type Beccles-Meppel.

In the National Institute of Public Health both series of typing phages have been used. Only few strains reacted with phages of the series serology II, but not with the corresponding phages with the serology VI. This might be explained by the lack of an antigen, that plays a part in the adsorption of these phages to the bacteria.

THE FELIX AND CALLOW SYSTEM.

Phage typing achieved practical significance when Craigie and Yen described a varietal sub-division of *S. typhi* which was obtained with adapted preparations derived from a single phage. This result opened the course for testing the principle of adaptability with *S. paratyphi* B also. Felix and Callow (1943) discovered a phage which only attacked part of the strains and would adapt itself to others. This phage has not, indeed, given the large number of different adaptations that could be obtained with the Vi-phage II of Craigie and Yen. Only four adapted preparations could be derived from the original phage 1 of Felix and Callow, namely 2, 3a and 3aI (which are identical), Jersey and lastly Beccles-Meppel, an adaptation found by Scholtens (1952), but which up to now has not been incorporated in the Felix and Callow system.

Five of the further phages of the Felix and Callow system appeared to be identical with the natural phages found in *S. paratyphi* B.

Felix and Callow (1943) first described types 1, 2, 3a, 3aI, 3b. Later types Jersey, Beccles, Taunton and Dundee have been described, and types 3a, 3aI and 3b subdivided into variations. Now the natural phages of bacteria have a particular value for the phage typing of the kind of bacterium by which they are produced (Scholtens, 1950, 1952, 1955, 1956).

The FELIX and CALLOW system, however, does not contain all the natural phages necessary for phage typing of these bacteria. Further natural phages have been found, that are good typing phages. In fact the addition of these phages to the system has established the parallelism between phage typing and the epidemiology that was lacking in the Netherlands.

THE NATURAL SYSTEM OF PHAGE TYPING.

The primary reason for seeking an extension of the system of FELIX and CALLOW was the discovery of an epidemiological entity, type Sittard, the strains of which were scattered over two different types on typing according to FELIX and CALLOW (SCHOLTENS, 1950, 1955). Needless to say this is very unfavourable for the aim of the method.

NICOLLE (in a personal communication) confirmed these findings, but writes (1957) that the results obtained in all Western European countries are excellent.

Before the natural system can be discussed, a remark must be made on the way the natural phages are demonstrated. Primarily natural phages may be isolated by filtration of a broth culture and by testing this filtrate on a sensitive strain. In this way, however, not all the natural phages that can be produced by the strains are found. When two strains are grown in mixed cultures natural phages will occur that are not found in the pure broth of either of the strains used. It would lead too far to discuss the complicated phenomena in the mixed cultures in detail; it may suffice that these natural phages sometimes derive from natural phages of low virulence, which are often set free only in mixed cultures and change and increase in virulence.

Before the natural system could be drawn up, fundamental insight in the pattern of phage resistance and sensitivity had to be gained, *viz.*, the distinction between the two kinds of phage reactions, the group reaction and the type reactions. By means of the group reactions *S. paratyphi* B is subdivided into groups or series of phage types. All strains of the types of a single group give the same pattern of group reactions. The group reactions are given firstly by the adaptations of phage 1 of FELIX and CALLOW, and secondly by phages found in the mixed cultures.

It can be seen in table 2 that all the types of group A react with the typing phages with the lytic spectrum d and h, and that the

TABLE 2. Natural grou

Group	Types according to FELIX and CALLOW	Natural types found by means of adaptations and the study of spontaneous lysogenicity					
			Gr				
			Adaptations of phage 1 of FELIX and CALLOW				
			1	2	3a 3aI	Jer-sey	Bee Mee
			Nomencla				
			1	2	3a	Jer-sey	
A	3b-most common	3b-most common	–	–	–	–	
	Beccles	Beccles-22	–	–	–	–	
	3b-variation 2	3b-variation 2	–	–	–	–	
	Dundee	Dundee	–	–	–	–	
	Taunton-the Hague	Taunton-the Hague	–	–	–	–	
M	3b-variation 1	3b-variation 1	–	–	–	–	
	Beccles	Beccles-Midwoud	–	–	–	–	
	B.A.O.R.	B.A.O.R.	–	–	–	–	
S	3a	"54"	–	–	(cl)	–	
	Beccles? 3aI?	"Sittard"	–	–	(cl)	–	
		"18"	–	–	(cl)	–	
	3aI-most common or Dundee	"87"	–	–	(cl)	–	
J	3a-Jersey?	"60"	–	–	cl	cl	
	Jersey	Jersey	–	–	(cl)	cl	
	3a-variation 3	3a-variation 3	–	–	cl	cl	
B.M.	Beccles	Beccles-Meppel	–	–	–	–	
B	3a-most common	3a-most common	–	–	cl	–	
	3a-variation 2	3a-variation 2	–	–	cl	–	
	3aI-variation 1–2	3a-variation 1–2	–	–	cl	–	
	3aI-most common	3aI-Schiedam	–	–	cl	–	
	3a-variation 4	3a-variation 4	–	–	cl	–	
	3aI-most common	3aI-Leeuwarden	–	–	(cl)	–	
	Taunton	Taunton-Kampen	–	–	(cl)	–	
2	2	2	–	cl	–	–	
Ungrouped types	1-most common	1-most common	cl	cl	(cl)	cl	

cl = confluent lysis.
(cl) = the reaction is not always, or even rarely present.
scl = semi confluent lysis.
* = plaques as dustlike particles, to be observed with the colony microscope.
+1, +2, +3 increasing number of plaques.

age types of *S. paratyphi* B.

Phagereactions														
actions						Type reactions								
ostranges of the phages found in the mixed cultures						Directly demonstrable type determining natural phages				Additional Phages		Directly demonstrable type determining natural phages found in the types		
e	d	c	f	h	b	I	IVb	IVa	VI					
of preparations in the system of FELIX and CALLOW														
Dundee						3b	Beccles	Taunton		B.A. O.R.	polyvalent anti-O phage			
–	cl	–	–	cl	–	cl	cl	cl	cl	cl	cl	–	–	–
–	cl	–	–	cl	–	–	cl	cl	cl	–	cl	I	–	–
–	cl	–	–	cl	–	cl	–	–	–	cl	cl	–	VI	–
–	cl	–	–	cl	–	–	–	–	–	–	cl	I	VI	–
–	cl	–	–	cl	–	–	–	cl	–	–	cl	–	–	IV
cl	–	cl	–	cl	–	cl	cl	cl	cl	cl	cl	–	–	–
cl	–	cl	–	cl	–	–	cl	cl	cl	(cl)	cl	I	–	–
cl	–	cl	–	cl	–	(cl)	–	–	–	cl	cl	–	VI	–
cl	cl	–	cl	cl	+1	cl	cl	cl	cl	cl	cl	–	–	–
cl	cl	–	cl	cl	+1	–	cl	cl	cl	(+1)	cl	I	–	–
cl	cl	–	cl	cl	+1	cl	–	–	–	(+3)	cl	–	VI	–
cl	cl	–	cl	cl	+1	–	–	–	–	–	cl	I	VI	–
cl	cl	–	–	scl	cl	cl	cl	cl	cl	cl	cl	–	–	–
cl	cl	–	–	scl	cl	–	cl	cl	cl	cl	cl	I	–	–
cl	cl	–	–	scl	cl	cl	–	–	–	cl	cl	–	VI	–
cl	cl	–	–	–	cl	–	cl	cl	cl		cl	I	–	–
cl	cl	–	–	cl	cl	cl	cl	cl	cl	cl	cl	–	–	–
cl	cl	–	–	cl	cl	cl	–	–	cl	–	cl	–	–	–
cl	cl	–	+1*	cl	cl	–	cl	cl	cl	cl	cl	I	–	–
cl	cl	–	–	cl	cl	–	–	–	cl	–	cl	I	–	–
cl	cl	–	–	cl	cl	+2	–	–	–	(cl)	cl	–	VI	–
cl	cl	–	–	cl	cl	–	–	–	–	–	cl	I	VI	–
cl	cl	–	–	cl	cl	–	–	cl	–	–	cl	I	–	IV
cl	cl	–	cl	cl		–	–	(+4*)	cl	–	cl	–	–	–
cl	cl	–	cl	cl	cl	–	–	(+4*)	cl	cl	cl	–	–	–

urvey of the natural grouping of a part of the phage types of *S. paratyphi* B. There are two inds of phagereactions, first the group-reactions which all types of one and the same group f phage types have in common, and second the typereactions which differentiate the types n the group. Group-reactions give: 1. the adaptations of phage 1 of FELIX and CALLOW and . bacteriophages found in the mixed cultures. Typereactions give the directly demonstrable, ype determining natural phages I, IVa, IVb, and VI.

three types of the next group (M) all react with the typing phages e, c, and h, and that the adaptation to type Jersey is a group reaction of group J.

The pattern of group reactions indicates the group. Now, the strains of the types from the same group have lysogenic properties in common e.g. all strains of all types of group A produce phages with a lytic spectrum e. (These phages are demonstrated best in mixed cultures with strains of types of group M. They originate from natural phages of lower virulence). These phages with lytic spectrum e attack all types except the types that may produce them i.e. except group A. They are important typing phages.

In a similar way all strains of all types of group M produce the phage lytic spectrum d, which attacks all types except the types that can produce it. In this way the strains belonging to types of the same group have not only phage reactions, but also lysogenic properties in common.

Now each group is subdivided by means of the same type reactions into phage types. For these reactions the natural phages that can be found in the pure broth cultures of the strains are utilised. These are the natural phages I, IV and VI¹). Phage I corresponds with the typing phage 3b of Felix and Callow, phage IV is found in two forms, IVa and IVb, that correspond with the typing phages Beccles and Taunton. Now in each series the types can be arranged in such a way, that an increasing content of natural phages is found, and a decreasing sensitivity for these phages.

This can be followed up in table 2. It can be seen in the last column of the table, that type 3b (most common) does not contain any of the latent phages I, IV or VI. Corresponding with the absence of these natural phages, a sensitivity to these phages is found. The next type Beccles-22 differs from type 3b in that it contains the natural phage I and is correspondingly insensitive to it. The next type, type 3b variation 2 possesses a natural phage VI. This natural phage confers an insensitivity on the strain not only for the phage VI itself, but also for the typing phages IVa and IVb, Taunton and Beccles of Felix and Callow.

In the next series a similar increase in content of natural phage is found, and parallel to this a similar decrease in phage sensitivity. Type 3b variation 1 does not contain one of the natural phages

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¹) Phage I corresponds with Burnet's group A of the *Salmonella* phages (1932). Phages IV and VI belong to group B.

I, IV or VI, and is sensitive to these typing phages. The next type, type Midwoud, or Beccles-Midwoud, differs from the former therein that it does contain the natural phage 1, and is resistant to it. It differs from type 3b variation 1 in the same way as type Beccles-22 differs from type 3b (common type). Next type is type B.A.O.R. It differs from type Midwoud and 3b-variation 1 in the same way as type 3b-variation 2 differs from Beccles-22 and 3b-common type. Then comes a series of new types, namely types "54", Sittard, "18" and "87".

The three types that react with the preparation adapted to type Jersey are grouped together. They differ in a similar way. Type "60" differs from type Jersey and from type 3a variation 3 in the same way as the types belonging to the groups discussed already differ from each other.

It is to be noted that all the strains of these three types have a biochemical character in common, as they do not ferment rhamnose whereas this sugar is fermented by most of the other groups. It is an important point that this group of strains, found to be related by means of the phage typing, have a definite bacterial property in common. It is clear that these series of phage types and the families of phage types of Dr Nicolle are of the same nature. Dr Nicolle is right when he suggests that there are more intimate bacterial factors that dominate these relationships.

Resuming we may say, that the types are characterised first by the phage reactions of the group they belong to, and that one group of lysogenic properties parellels these reactions, and further by their type reactions and that a second group of lysogenic properties parallels these latter reactions. In this way a typing system is drawn up wherein, just as in the case of *S. typhi*, all strains of each type possess the same lysogenic properties.

The system relies in the first place upon phage reactions; in view of the parallelism between the latter and the lysogenicity of the types an examination of the bacteria for natural phages may be made, in order to characterise the types, all the more so when the diagnosis remains doubtful the phage reactions being taken into account only.

TYPES FROM THE FELIX AND CALLOW SYSTEM WHICH HAVE NOT BEEN PLACED IN THE NATURAL SYSTEM OF PHAGE TYPES.

Various types from the Felix and Callow system have not been placed in the natural system of phage types. These are the types which have been registered as "unstable" by Felix and Callow, also the d-tartrate positive types.

a. Unstable types.

Felix and Callow (1951) distinguish several sub-types in their system on the ground that these strains do not react with their typing phage "Dundee". These are sub-types 3b-variation 1, 3a-variation 1 and 3aI-variation 3. The typing phage "Dundee" of Felix and Callow corresponds with the bacteriophages with the lytic spectrum d and serology II from the natural system. This phage reaction represents an important group reaction by which types of group M, *viz.*, 3b variation 1, Midwoud and B.A.O.R. can be distinguished from the types of the other groups as they do not react with this phage and the types of all the other groups do. The typing phages c have a lytic spectrum which is directly opposed to lytic spectrum d; they react only to types from group M. Group M can thus be characterised by the two group reactions described above, the one whereby types of the group are specifically attacked, and the other whereby all types are attacked except those of group M. The differences in sensitivity observed here are very great. Neither will a phage with lytic spectrum d lyse types of group M in a 1000 times critical test concentration. A specific preparation of the phage with lytic spectrum c could be obtained with a critical test concentration of 10^{-3}, which undiluted attacked none of the other types.

Whilst these three types, which do not react with the typing preparation "Dundee" of Felix and Callow, can be very clearly distinguished by means of preparations with lytic spectrum c and d, the existence of sub-types 3a variation 1 and 3aI variation 3 could not be confirmed.

The following reasons can be adduced for this:

1. It is true that some strains of types 3a-common type and 3aI-common type react poorly or not at all with typing phage "Dundee". Nor can this insensitivity be compared quantitatively with the specific insensitivity of the strains of group M to this preparation. Strains usually do react in a tenfold of the critical test concentration. Consequently these types cannot easily be separated from the types 3a-common type and 3aI-common type.

2. There is no epidemiological relation between the cases from which the strains reacting poorly were isolated. This was not only the

experience in the Netherlands; in fact among strains from a single epidemic which had been sent on by Dr BEUMER from Belgium, strains were present which had been diagnosed by him as 3aI-common type as well as strains which had shown the reaction of 3aI-variation 3 in his laboratory. According to the natural system these strains all reacted as 3aI-Leeuwarden.

3. The type strains which FELIX made available did not differ from strains of types 3a-common type and 3aI-common type.

4. Not all phages with lytic spectrum d show this incomplete reaction. The phages with lytic spectrum d and serology VI are completely virulent to all strains of these types.

5. The strains of types 3a-common type and 3aI-common type which are less sensitive to typing phage "Dundee" do not show the complementary positive reaction with the typing phage c by which strains of group M are characterized.

Thus none of the types which are recorded in the FELIX and CALLOW system as "unstable" have been incorporated in the system.

b. D-tartrate positive types.

The strains of types 1, variation 3 and 4, and 3b, variations 3 and 4, of FELIX and CALLOW are d-tartrate positive and belong therefore to *Salmonella java*. *S. java* causes a different syndrome and has a different epidemiology than *S. paratyphi* B. To the epidemiologist the diagnosis of *S. java* is thus of greater importance than placing the strain in one or other phage type. Moreover, the great majority of *S. java* strains are untypable; also they form a considerable percentage of the untypable strains.

This agrees with the findings of BRANDIS (1955). Also, on the single occasion that phage reactions of these strains agreed with those of one of the types of *S. paratyphi* B in the Netherlands, no phage type diagnosis was communicated to the epidemiologist. If we wish to phage type this very varied group of bacteria a special system of phage types for *S. java* would have to be set up.

The FELIX and CALLOW system contains therefore eighteen different types of *S. paratyphi* B whose existence can be confirmed. Types 1 variation 1 and 1 variation 2 that have only rarely been met with are included in these eighteen types.

TYPES WHICH APPEAR IN THE NATURAL SYSTEM, BUT NOT IN THE FELIX AND CALLOW SYSTEM.

Types "22", Taunton-Den Haag, Midwoud, "54", Sittard, "18", "87", "60", Beccles-Meppel, 3aI-Schiedam and 3aI-Leeuwarden do not appear in the FELIX and CALLOW system. In the natural system, 9 more types are distinguished than in the FELIX and CALLOW system (Table 3).

These types are first determined by phage reactions for which, besides the adaptations of phage 1 of FELIX and CALLOW, the natural phages from the bacterial species are selected. The strains of the types thus determined possess the same lysogenic properties; in consequence the lysogenic properties may be used as characteristics of the types. The study of lysogenic properties can be used as a check on the homogenicity of the type. A type is a collection of strains which (within certain limits) possess the same lytic spectrum and lysogenic properties; these two groups of properties being interdependent.

Table 2 contains, unlike the table in a former publication (SCHOLTENS, 1956) type 2 of FELIX and CALLOW as a representative of a new group, as this type is indicated by an adaptation (group reaction). Type 1 of FELIX and CALLOW has been added as an ungroupable type. Eventually this may also be the case with subtypes 1 variation 1 and 1 variation 2.

Type 3a variation 2 has taken its place in the system. The type strain B 4097 is sensitive to typing phage VI and insensitive to the typing phages IVb and IVa (Beccles and Taunton of FELIX and CALLOW). In this respect this type corresponds with type 3aI Schiedam, from which, however, it differs by its sensitivity to and its absence of natural phage 1.

Besides through the addition of new series of phage types ¹) and the finding of the missing types of the incomplete series the system can be extended by new group reactions that subdivide all types of a definite series. Here group B is particularly noticeable as this group is least characterised by specific reactions.

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¹) While this paper was in press, Dr. RISCHE found two new types: a type M_6 from group M that showed the group reactions of group M, but did neither react with the type determinating phages, nor with phage B.A.O.R. of the FELIX and CALLOW system, and a type J_7, that gave the group reactions of type Jersey and type reactions similar to type Taunton. These types will be described elsewhere.

TABLE 3.

Correction of diagnoses made with the Felix and Callow system.

		Phage reactions														
Diagnoses Felix and Callow	Diagnoses natural system	1	2	3a 3aI	3b	Jersey	Beccless	Taunton	B.A. O.R.	Dundee	e	c	f	h	B.M.	VI
Dundee	Dundee	–	–	–	–	–	–	–	–	cl	–	–	–	cl	–	–
	Taunton	–	–	–	–	–	–	–	–	cl	cl	–	–	cl	–	–
	"87"	–	–	–	–	–	–	–	–	cl	cl	–	cl	cl	–	–
	3aI-Leeuwarden	–	–	–	–	–	–	–	–	cl	cl	–	–	cl	–	–
	atypical strains	–	–	–	–	–	–	–	–	cl	cl	–	–	cl	–	–
3aI-common type	3aI-Leeuwarden	–	–	cl	–	–	–	–	–	cl	cl	–	–	cl	–	–
	3aI-Schiedam	–	–	cl	–	–	–	–	–	cl	cl	–	–	cl	–	cl
	"87"	–	–	cl	–	–	–	–	–	cl	cl	–	cl	cl	–	–
Beccles	"22'	–	–	–	–	–	cl	cl	(cl)	cl	–	–	–	cl	–	cl
	Midwoud	–	–	–	–	–	cl	cl	(cl)	–	cl	cl	–	cl	–	cl
	Beccles-Meppel	–	–	–	–	–	cl	cl	(cl)	cl	cl	–	–	–	cl	cl
	Sittard	–	–	–	–	–	cl	cl	(cl)	cl	cl	–	cl	cl	–	cl
3aI-variation 1 – 2	3aI-variation 1, 2	–	–	–	–	–	cl	cl	(cl)	cl	cl	–	–	cl	–	cl
	Sittard	–	–	–	–	–	cl	cl	(cl)	cl	cl	–	cl	cl	–	cl

The diagnoses made with the natural system corresponded to epidemiological findings.

PRACTICAL RESULTS.

More types are found in the natural system than in the FELIX and CALLOW system. It may be of some importance that a type from one system has been subdivided into two types in a further system, but here, in both cases a parallelism with the epidemiological findings will be found (Table 3).

More serious harm to the method results when the strains from a single outbreak are given different diagnoses; in other words, when the cases of a single epidemiological entity are scattered over different types.

The following examples are presented which are brought together in table 3.

It has been found, that strains have been diagnosed wrongly as "Dundee" in the FELIX and CALLOW system. In this system these strains react with the typing phage "Dundee" only. In the natural system strains of type "Dundee" show another characteristic reaction, they do not react with the typing phage e, and in this way higher demands are made in the natural system on the diagnosis "Dundee".

Now initially strains of type Taunton have been found, that did not react with the typing phage Taunton and were typed "Dundee" with the FELIX and CALLOW system (BEUMER, RISCHE, personal communication). These strains did, however, react with the typing phage e, and therefore they could not be typed in the natural system by means of phage reactions. By the demonstration of the natural phage IV in these strains they can be typed as Taunton.

Further strains of a new type, type "87" from the natural system are typed sometimes as "Dundee" and sometimes as 3aI-common type (RISCHE, personal communication). In the natural system these strains are diagnosed "87" with the typing phages e and h. This is a rare type, but it is precisely in the case of rare types that phage-typing may be successful. RISCHE found three strains of this type in Rostock. He typed one of them "Dundee' according to FELIX and CALLOW and the other two "3aI" and thus led the epidemiologists astray; with the diagnosis "87" he was successful. Further it has been found that strains of type 3aI-common type may fail to show the reaction with the typing preparations 3a and 3aI. They are typed as "Dundee" in the FELIX and CALLOW system.

Then there are atypical strains, that react as "Dundee" in the FELIX and CALLOW system. So in a certain family the children had mild diarrhoea and the mother suffered from a febrile disease affecting the hip joint. The strains isolated reacted as "Dundee" in the system of FELIX and CALLOW. When examined by means of the natural system they were untypable, an examination of the natural phages showing that they were atypical strains as they contained atypical natural phages. Here the diagnosis "untypable" was the best one.

On the other hand, it was observed in the case of a carrier left over from an epidemic caused by type "Dundee", that type "Dundee" can react, albeit very seldom, with the preparations 3a-3aI of FELIX and CALLOW. None of all the strains from this epidemic reacted either with the typing phage e from the natural system, or with preparations 3a-3aI.

A further epidemiological entity, that is scattered over two types in the FELIX and CALLOW system, is type "Sittard". In 1955 an epidemic of 200 cases, the strains of which could not be typed according to the system of FELIX and CALLOW was described (SCHOLTENS, 1955). Now we find every year ten to fifteen strains of this type. The strains of this epidemiological entity reacted partly as 3aI, partly as Beccles. The same strain reacted as "Beccles" on one typing, and as 3aI at another time. The same strains were sent to Dr FELIX and to Dr NICOLLE; Dr FELIX typed them Beccles, and Dr NICOLLE typed them 3aI. In the natural system these strains are grouped with the typing phage f and the presence or absence of the reactions 3a-3aI is immaterial for the diagnosis. It is clear that the scattering of the strains of this epidemiological entity over different types impairs the agreement with the epidemiology.

The larger the material examined the more cases were found wherein a correction of the diagnosis with the additional typing phages brought about a more complete parallelism with the epidemiological findings.

SUB-DIVISION OF TYPE BECCLES OF FELIX AND CALLOW.

A few points must be made here on the type Beccles of FELIX and CALLOW. There are three types in the natural system which are diagnosed as "Beccles", Beccles 22, Beccles-Midwoud and Beccles-Meppel, outbreaks of these types being described by NICOLLE and co-workers (1955) and by SCHOLTENS (1950, 1952, 1955, 1956), whilst, moreover, some of the strains of type Sittard are diagnosed as Beccles. These are four types, well-defined by specific reactions.

Not all strains, however, of type Beccles of Felix and Callow can be classed with these types. Rare strains found by Dr Rische react as Beccles according to Felix and Callow, but belong to none of these four types. They agree with types 3aI variation 1, 2 except that they do not react with typing phages 3a-3aI of Felix and Callow. However, they yielded subcultures, that reacted as type 3a (common type). Further epidemiological research will be needed to settle whether a new sub-type of type Beccles must be established here. Perhaps these strains, however, must also be discussed from the point of view set forth below.

Unreliability of phage reactions 3a-3aI in the Felix and Callow system.

If we survey the results described above it is noticeable that the reactions with typing phages 3a-3aI are those which every time fall short in the Felix and Callow system. These may or may not be present in some types.

This is the case with types Jersey and Taunton in the Felix and Callow system. Here the diagnosis is based, however, on another phage reaction. A similar variability in these reactions exists in other epidemiological groups; here, however, types are established by Felix and Callow in which the diagnosis depends on the unreliable reaction 3a-3aI. In the natural system reliable phage reactions have been found which when added permit a diagnosis with the result that the presence or absence of phage reactions 3a-3aI is just as irrevelant as with types Jersey and Taunton (Types Sittard, "87", 3aI-Leeuwarden).

Bearing in mind the unreliability of reactions 3a-3aI we can appreciate the possibility of strains which belong epidemiologically to strains of types 3a variation 1 or 2, but which do not react with preparations 3a-3aI. Their reactions agree with the Beccles strains of Rische mentioned above. Here further epidemiological research is desirable.

Demonstration of the natural phage in the bacterium; the untypable strains.

The presence of a natural phage in bacteria is always coupled with a resistance to this phage; this does not apply to the opposite. Whenever a strain is resistant to a natural phage this phage is not necessarily demonstrable in the strain. These exceptions are generally found in all cases of a same explosion.

However, a strain of type Taunton was found which did not contain the natural phage I (though it did contain the natural phage IV). This strain was first discovered on a patient on whom strains with the natural phages I and IV had previously been found. All other strains from the same epidemic contained the natural phages I and IV. Wider epidemiological experience concerning the significance of these exceptional cases would be desirable.

There are, however, cases where the demonstration of a natural phage is the only sure method by which a type diagnosis can be made.

These are, firstly, the strains already discussed of type Taunton (sometimes, according to Felix and Callow, diagnosed as "Dundee", but mostly found "untypable"). These strains can be diagnosed as Taunton by the demonstration of the natural phage in the bacteria. They react with the combined phages 0_{1-2-3} of Felix and Callow and sometimes more or less strongly with typing phage "Dundee" of Felix and Callow. They contain the natural phage IVb. There are, however, also strains which show a complete lack of phage reaction, but which may be typed by demonstration of the natural phage; but these are strains of type "Taunton", where the natural phage IVa is found. These strains were isolated in carriers where, previously, normal strains of type "Taunton" had been isolated.

Moreover, strains are found which contain a-typical natural phages. These are, firstly, the strains already mentioned above which according to Felix and Callow should have been typed as "Dundee", but which reacted with typing phage e. Dr Rische sent a similar strain. This was at the same time isolated with a strain from type B.A.O.R. from the same patient. It appeared to contain a natural phage which serologically and as far as plaque form is concerned agreed with phage VI but, in contrast with the usually found phage VI, also attacked type Taunton. The same natural phage was observed in a strain of type "87" which was isolated in Utrecht. Further strains were found that showed the phage reactions of type "Dundee" in the Felix and Callow system and reacted with phage e of the natural system. These strains produced natural phages that were serologically similar to the phages VI from the natural system, but differed in thermostability from the latter. The strains caused gastroenteritis. They will be described extensively elsewhere.

These sporadic abnormal strains were untypable in the natural system. This is also the best information for the epidemiologist.

Summary and Conclusions.

As the results obtained with the system of Felix and Callow were not in accordance with epidemiology, this typing scheme has had to be changed. Use was made of the study of lysogenicity to give practical effect.

A typing scheme was obtained in which types were determined by means of phage reactions. The natural phages of *S. paratyphi* B as well as the adaptations of phage 1 of Felix and Callow were used for these phage reactions. In the thus composed typing scheme the strains of the same type possess the same lysogenic properties; in consequence, the lysogenic properties become of value for a differentation of types.

This natural system was compared with the Felix and Callow system by members of the first Subcommittee on *Salmonella paratyphi* B in the Netherlands, Belgium and Germany.

Some natural phages of *S. paratyphi* B are already used in the system of Felix and Callow. The natural system contains, however, still more phages, by means of which a better definition of types becomes possible. This becomes particularly noticeable in type "Dundee"; several strains which were typed as "Dundee" in the Felix and Callow system seem to belong to other epidemiological entities. Moreover, it seems that we cannot regard as reliable the reactions with typing preparations 3a-3aI in the Felix and Callow system, to which in many cases decisive diagnostic value has been ascribed. In the natural system these preparations have no longer decisive diagnostic value due to the addition of other phage preparations. The types which are recorded as unstable in the Felix and Callow system have not been admitted to that system. On the other hand, nine types have been given which do not exist in the Felix and Callow system.

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ON THE MODE OF ACTION OF DIALKYLDITHIOCARBAMATES ON MOULDS AND BACTERIA

by

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In the course of the last few years we have obtained a better insight into the mode of action of an important group of agricultural fungicides, the dialkyldithiocarbamates, and we should like to give here a general picture of the views we hold at present. Whereas the dialkyldithiocarbamates differ in their mode of action from the bisdithiocarbamates (17), we have found that there is a close parallel in the behaviour of dialkyldithiocarbamates, 8-hydroxyquinoline and pyridine-2 thiol-N-oxide (19,21).

When testing growth of a large variety of moulds on glucose mineral salts agar of pH 7.0, made up with tap water and containing different amounts of sodium dimethyldithiocarbamate (NaDDC) or tetramethylthiuram disulphide (TMTD, the oxidation product of NaDDC), one will find that according to their behaviour the moulds can be roughly divided into three groups (Table 1).

Group I: complete growth inhibition by *ca.* 0.2 p.p.m. of NaDDC. This picture of "sensitive moulds" is shown by *Glomerella cingulata* (21), *Botrytis allii*, *Botrytis cinerea*, *Ceratostomella ulmi*, *Glomerella fructigenum*, *Sclerotinia fructicola* and the yeast *Rhodotorula glutinis*.

Group II: growth is inhibited by low concentrations of NaDDC, *e.g.* 0.5-2 p.p.m. ("first zone of inhibition") but returns again at higher concentrations: 5, 10 and 20 p.p.m. ("zone of inversion growth"); inhibition follows again from 50 p.p.m. onwards ("second zone of inhibition"). The moulds showing this unusual pattern we have called "moulds with inversion growth". Examples: *As-*

TABLE 1.

Inhibition of moulds by NaDDC on a glucose mineral salts solution made up with tap water.
(aneurin and biotin added when necessary).

	p.p.m. NaDDC										
	0	0.05	0.1	0.2	0.5	1	2	5	10	20	50
Group I *Glomerella cingulata* type	+	+	+	—	—	—	—	—	—	—	—
Group II *Aspergillus niger* type	+	+	+	+	—	—	—	±	+	+	—
Group III *Fusarium oxysporum* type	+	+	+	+	+	+	+	+	+	+	—

pergillus niger, Asp. oryzae, Asp. ruber, Alternaria solani, Alt. tenuis, Cladosporium cucumerinum, Clad. herbarum, Pen. brevicompactum.

Group III: growth is inhibited from *ca.* 50 p.p.m. of NaDDC upwards. Examples of these insensitive moulds are: *Fusarium oxysporum, Pen. expansum, Asp. fumigatus, Asp. nidulans, Asp. ochraceus, Asp. versicolor, Fusarium avenaceum, Fus. bulbigenum, Fus. coeruleum* and *Pen. citrinum.*

A behaviour intermediate between these groups can also be found: Slow growth in the zone of inversion growth [*e.g. Pen. italicum* (18) and *Saccharomyces cerevisiae*] or in the first zone of inhibition is seen then. The phenomenon of inversion growth has already been described by DIMOND, HEUBERGER and STODDARD (8) in 1941 for spore germination tests with *Macrosporium sarcinaeforme* and TMTD where they found a double maximum curve.

With the exception of *A. niger* the action of all moulds studied is independent of the pH value (16). This mould belongs to group II at pH above 6.5, whereas below a pH of *ca.* 6.5 a first zone of inhibition is not seen (group III).

A further study of these three groups has given us a better insight into their interrelationships and at the same time into the relation of growth inhibition by NaDDC and other dithiocarbamates, by 8-hydroxyquinoline (oxine) and by pyridine-2-thiol-N-oxide (PTO).

First zone of inhibition:

Our starting point has been the observation that on malt agar of pH 7.0 and on agar with 1% "casamino acids" (Difco) *Aspergillus niger* appears to grow in up to 20 p.p.m. of NaDDC, the first zone of inhibition which is shown on glucose agar, now being absent (16). Also the moulds of group I appear far less sensitive on these media than on glucose agar. For the insensitive moulds (group III) the medium does not influence the results. An analysis of the antagonistic activity of some probable constituents of "casamino acids" showed us (16) that of 18 amino acids, 9 growth factors, 4 purines and 2 pyrimidines, L-histidine is the only compound that is able to counteract NaDDC at pH 7.0. This effect is far more pronounced at this pH value than at pH 4, where cysteine was found to antagonize, as was also reported by Smale, Cox and Sisler (31). Thus the histidine contained in malt is held responsible for the observed difference in growth response on malt agar as compared with that on glucose agar. Earlier results of Klöpping and van der Kerk (25) on the activities of dithiocarbamates are quite comparable to our present results if we consider that their experiments were carried out on malt agar of pH *ca.* 6.0.

Histidine as well as certain other imidazole derivatives (18) appeared to be competitive antagonists of NaDDC for moulds of group I and II, but only in the region up to 20 p.p.m. of NaDDC. At 50 p.p.m. and higher concentrations histidine did not show any effect. These results suggested strongly that for these two groups the mode of action of NaDDC at low concentrations differs from its mode of action at higher concentrations (from 50 p.p.m. onwards).

The role of metals:

We had found earlier that also oxine was less active on malt agar than on glucose medium. As it appeared that here too histidine as well as certain other imidazole derivatives were antagonists, we suggested a common factor in the mode of action of NaDDC and oxine.

It is a well-known fact that dialkyldithiocarbamates, oxine as well as histidine form complexes with metals. Moreover, the mode of action of oxine on bacteria was found by Albert (1) to be connected with complex formation of oxine with Fe and Cu; bactericidal activity was found to coincide with the presence in the medium of oxine and metal in a 2 : 1 ratio, whereas toxicity was less when either oxine or the metal was present in excess. Meanwhile Block (5) has confirmed these findings for fungi. This suggested strongly that metals might also play a role in the fungitoxic action of NaDDC. A further confirmation was obtained by an investigation of the metal-binding ability of the various imidazol derivatives, which are active antagonists. We found that when Cu was taken as the metal, there was indeed a strong correlation between this property and the antagonistic activity (unpublished).

The role of metals was then further investigated especially with regard to the work of Goksøyr (9) He stressed the importance of the two complexes of Cu and DDC in the inhibitory action of NaDDC on *Saccharomyces cerevisiae.* An equilibrium exists between these two possible complexes of Cu^{II} and DDC in the following way:

$$\underset{1:1\ \text{complex}}{\mathrm{CuDDC^+}} \ \underset{+\mathrm{Cu}}{\overset{+\mathrm{DDC}}{\rightleftarrows}}\ \underset{1:2\ \text{complex}}{\mathrm{CuDDC_2}}$$

The 1 : 1 complex is water soluble and its concentration can be determined spectrophotometrically. The 1 : 2 complex is extremely insoluble (see later). Goksøyr studied the combined effect of NaDDC and metal salts on the oxidation of acetate and glucose. A maximum in inhibition of acetate oxidation was found to coincide with a maximum in the amount of 1 : 1 complex present (namely in media containing 6.25 p.p.m. $CuSO_4.5\ H_2O$ and 0.9-1.5 p.p.m. NaDDC). At higher NaDDC concentrations the 1 : 1 complex is turned into the 1 : 2 complex and there was no inhibition of acetate oxidation. Also in a medium without added Cu the reversal phenomenon was seen in the inhibition of acetate oxidation; it was ascribed to the presence of NaDDC chelated with traces of metals adhering to the yeast cells. Under these latter circumstances the effect of NaDDC on growth was also studied and strict correlation between growth inhibition and inhibition of acetate oxidation was found, both showing a double maximum curve. Although no growth experiments were carried out with NaDDC and added Cu, it is inferred by Goksøyr from the above experiments that growth inhibition in the first zone is due to 1 : 1 complex. Inversion growth occurs when all 1 : 1 complex has been transformed into the non-toxic 1 : 2 complex.

The role which Cu appeared to play urged us to apply henceforth basal media with an extremely low Cu content. To this end we used (21) a glucose mineral salts solution (pH 7.0) with a Cu content not exceeding 0.01 p.p.m.

The Cu content of the agar which we had used for our media was only very low. We had, however, generally washed this agar and it was now found that by washing with tap water the Cu content of agar increases considerably. An argument against using unwashed agar was the fact that for as yet unknown reasons, the same amount of Cu was far more effective here than in liquid media.

By comparing growth results in liquid shaken medium (Table 2) to which Cu had been added, with the u.v. absorption spectra of similar media (20), we could confirm that for *A. niger* the first zone of inhibition coincided closely with the presence of certain amounts of 1 : 1 complex of Cu and dimethyldithiocarbamate. This amount is not yet reached in media with 3 p.p.m. of copper sulphate in con-

TABLE 2.

Effect of addition of copper sulphate on growth inhibition of *Aspergillus niger* by NaDDC.

Shake culture in glucose mineral salt solution; glass-distilled water; pH 7.0; aneurin and biotin added; 3 days 24°.

p.p.m. $CuSO_4.5H_2O$ added	p.p.m. NaDDC								
	0	0.2	0.5	1	2	5	10	20	50
0	+	+	+	+	+	+	+	+	—
3	+	+	+	+	+	+	+	+	—
10	+	+	—	—	—	±	+	+	—

trast to those with 10 p.p.m. copper sulphate (*cf.* also Table 4). With increasing NaDDC concentration the 1 : 1 complex is turned into the very insoluble 1 : 2 complex and toxicity disappears. When excess of NaDDC is present — inevitably together with a certain amount of 1 : 2 complex — toxicity returns again, but in this case it is due in our opinion to the free dithiocarbamate ion (*cf.* also SMALE (32)), and not to zinc or iron dithiocarbamates as GOKSØYR (9) suggests for his experiments with yeast. These latter compounds hydrolyze to a great extent. Moreover, the amount of free ions of these metals is greatly reduced due to the presence of other complexing agents as *e.g.* the phosphate ions.

To produce the first zone of inhibition Cu clearly is required. Of other heavy metals tested (Fe, Co, Mn, Zn) only Co appeared to have some action which, however, is less than that of Cu (20).

The action of CuDDC and $CuDDC_2$ on moulds of the three groups:

The fact that CuDDC is toxic in contrast to $CuDDC_2$ was remarkable with respect to ALBERTS finding that $Cuox_2$ was more toxic than Cuox. A further study shed more light upon this discrepancy. When in our Cu-free culture solution (21) *G. cingulata* was grown in the presence of different amounts of NaDDC and copper sulphate (Table 3), no inversion growth was witnessed at any combination of NaDDC (up to 50 p.p.m.) and $CuSO_4$ (up to 10 p.p.m.).

It appeared to us that the difference in response between *A.niger* and *G.cingulata* might be understandable if *G.cingulata* were sensitive to $CuDDC_2$ in contrast to *A.niger* which is not inhibited even by high amounts. This supposition proved to be true as *G.cingulata* was inhibited by 0.05 p.p.m. of $CuDDC_2$, 0.02 p.p.m. still giving growth.

TABLE 3.

Effect of addition of copper sulphate on growth inhibition of *Glomerella cingulata* by NaDDC.

Shake culture in glucose mineral salt solution.

p.p.m. copper sulphate added	minimal inhibiting conc. of NaDDC (p.p.m.)
0	0.1
0.03	0.05
0.1	0.1
0.3	0.1
1	0.2
3	0.2

TABLE 4.

Amount of CuDDC present (p.p.m.) in the medium (1% glucose, 0.5% K_2HPO_4, 0.1% $(NH_4)_2SO_4$, 0.05% NaCl, 0.05% $MgSO_4$, glass distilled water; pH 7.0).

p.p.m. $CuSO_4.5H_2O$ added	p.p.m. NaDDC								
	0.02	0.05	0.1	0.2	0.5	1	2	5	10
0									
0.03	0.01	0	0						
0.1	0.02	0.03	0.02	0					
0.3	0.02	0.04	0.05	0.04	0	0			
1	0.02	0.05	0.1	0.1	0.1	0.06	0		
3	0.02	0.05	0.1	0.2	0.2	0.2	0.1	0	0
10	0.02	0.05	0.1	0.2	0.4	0.34	0.32	0.30	0.2

——— Inhibition pattern of *Glomerella cingulata*.

--------- ,, ,, ,, *Aspergillus niger*.

The solubility of this compound is only *ca.* 0.01 p.p.m. [(15), *cf.* Table 8]. Probably this concentration is just inhibitory, but the rate of dissolution is slow; therefore somewhat more seems to be required to build up quickly an inhibitory concentration.

The amounts of CuDDC and $CuDDC_2$ present in each instance of our experiments could be calculated (Table 4, 5) starting from the known values for the stability constants for the complexes and the solubility.

TABLE 5.

Amount of $CuDDC_2$ present in solution (p.p.m.) in the medium.

p.p.m. $CuSO_4.5H_2O$ added	p.p.m. NaDDC				
	0.02	0.05	0.1	0.2	0.5
0					
0.03	S	S	S	S	S
0.1	0.002	S	S	S	S
0.3	0.001	0.004	S	S	S
1		0.001	0.005	S	S
3			0.001	S	S

——— Inhibition pattern of *Glomerella cingulata*.
S = saturated solution of $CuDDC_2$ (*ca*. 0.01 p.p.m.).

In the calculations the partial hydrolysis of Cu^{++} ions at pH 7 was taken into account. No allowance was made for the action of other complexing agents, but since their concentrations are small, it is assumed that their influence will be negligible. Of course the calculated values of the Tables 4 and 5 are approximate. A comparison of these with Table 2 confirms that *A.niger* is not inhibited by $CuDDC_2$, but by the 1 : 1 complex, namely when the amount of this latter substance surpasses *ca*. 0.3 p.p.m.; this leads to the first zone of inhibition. For *G.cingulata*, however, $CuDDC_2$ had proved to be toxic. At all Cu concentrations sufficient $CuDDC_2$ seems to be present to explain growth inhibition, though at several instances the concentration of the 1 : 1 complex may at the same time be inhibitory as well (15). This cannot be proved since sufficient $CuDDC_2$ is invariably present to explain inhibition. Yet, similar experiments with diethyldithiocarbamate (see later) gave evidence that the 1 : 1 complex will be inhibitory as well. Inhibition in flasks without added Cu must be due to complex formation of dithiocarbamate with Cu present in the medium or adhering to the cells.

The difference between moulds with and without inversion growth (groups II and I) thus seems to be solely that the former are somewhat less sensitive than the latter so that a toxic concentration of the very insoluble compound $CuDDC_2$ cannot be reached. Further confirmation will be given below.

The distinction between group II and III equally is not fundamental. Moulds of the latter group do in fact also show a first zone of inhibition – followed by inversion growth – if only sufficient Cu is added. For instance with *F.oxysporum* on glucose agar when 10 p.p.m. of copper sulphate is added. For *A.niger* only 1 p.p.m. of copper sulphate is required to give the same effect on agar (20). Thus the difference between these groups appears to be a gradual one which means that the distinction in two groups is more or less deliberate. It was only on our, arbitrarily chosen, glucose agar medium made up with tap water and inevitably containing a certain amount of Cu that we could distinguish these two groups.

In view of the three types of response to NaDDC, it seems a happy coincidence that TMTD or the dimethyldithiocarbamates can be used at all as a fungicide. If the solubility of $CuDDC_2$ had only been slightly less, these compounds would have proved completely useless. We now know several important plant parasites which are sensitive to $CuDDC_2$ as *e.g. Venturia inaequalis*, and which can thus be combatted by dithiocarbamates; *Phytophthora infestans*, another ill-famed plant parasite, cannot be combatted by these compounds; $CuDDC_2$ in fact, was here not inhibitory *in vitro*.

Antagonists:

For the second zone of inhibition (NaDDC content 50 p.p.m. or more) we know no antagonists. But in the first zone of inhibition of *A.niger* growth can be restored (20): a) by the addition of agents that bind Cu more readily than NaDDC does, *i.e.* by higher dithiocarbamates, or b) by addition of a large excess of agents that bind Cu less readily than NaDDC, *i.e.* ethylene diaminetetraacetic acid (EDTA) or c) by certain imidazole derivatives. The latter bind Cu less readily than NaDDC and EDTA but they are believed to compete with the enzyme for the toxic 1 : 1 complex by formation of imidazole: Cu : DDC complexes. As exposed above the antagonistic activity of imidazole derivatives runs closely parallel to their Cu-binding ability. Also the fact that the antagonistic activity of imidazole derivatives decreases with lowering of the pH is an argument for metal binding being involved.

In general the pH dependence of the action of the fungitoxic agents as well as of the antagonists is in accordance with expectations. Dithiocarbamic acids which are completely ionized at pH about 7 show no effect of pH, whereas oxine and imidazole derivatives which are partially ionized do show the effect.

The same compounds are also antagonistic for other moulds; for those of group II in the first zone of inhibition; in group I up to about 20 p.p.m. of NaDDC (21).

α-Keto acids antagonize in the first zone of inhibition, but only when *A.niger* is used (18). We cannot give an explanation for this.

anomalous behaviour. Yet, on the other hand it seems possible that the remarkable fact, that this same mould shows no first zone of inhibition at low pH values, could be due to a production of α-keto acids by spores and mycelium leading to antagonism of NaDDC.

In general we can say that for moulds the action of NaDDC in various media is determined by the Cu content of the medium and by the histidine content of organic additions. The barrier of 50 p.p.m. of NaDDC can, however, not be surpassed since from here onwards the free dithiocarbamate ion is inhibitory.

Action of NaDDC on bacteria:

Though Gram+ bacteria seem to be very sensitive to NaDDC in contrast to Gram— bacteria, further confirmation of this is required. We found that the inhibition of *Bac.subtilis* by NaDDC follows the same pattern as that of the fungi of group I; there is no inversion growth. Complete inhibition is obtained by *ca.* 0.2 p.p.m. of NaDDC, and Cu probably is the only metal involved in inhibition.

Homologues of NaDDC:

If we now turn to the higher dithiocarbamates we find that the ethyl compound does not show a first zone of inhibition with *A.niger*; this mould is only inhibited by 50 p.p.m. of the diethyl compound irrespective of the amount of Cu present (21). On the other hand *G.cingulata* shows (21) with diethyldithiocarbamate more or less the pattern of *A.niger* with NaDDC (see Fig. 2). This difference between the dimethyl and the diethyl compound can be fully explained by the fact that the solubility of the 1 : 2 complex with Cu is far less for the latter compound than for the first namely only 0.002 p.p.m. (15); *G.cingulata* obviously is not completely inhibited by this concentration (Table 8). Spectrophotometric measurements have shown that the first zone of inhibition coincides with the presence of maximal amounts of 1 : 1 complex. However, because of the low solubility of the 1 : 2 complex the concentration of 1 : 1 complex present (*ca.* 0.15 p.p.m. when 0.2-5 p.p.m. diethyldithiocarbamate are combined with 10 p.p.m. copper sulphate) is less than the corresponding concentration when NaDDC is used. This fact explains why *A.niger* does not show a first zone of inhibition with the diethyl compound.

Because the 1 : 1 complex of the diethyl compound is toxic for *G.cingulata* we suggested above that the dimethyl complex is toxic too. It seems impossible, however, to tell which of the two 1 : 1 complexes is the most toxic, since the toxic $CuDDC_2$ always accompanies the CuDDC.

Bac. subtilis follows again the pattern of *G. cingulata* showing a first zone of inhibition and a zone of inversion growth. CRUICKSHANK (7) in a paper on the differentiation of three *Brucella* species describes that, when a disc containing diethyldithiocarbamate is placed on a plate which then is incubated with the bacteria, *Brucella melitensis* and *Br. abortus* develop around the disc a ring of growth between two rings of inhibition, whereas *Br. suis* shows only one large zone of inhibition. This points to the same differentiation as we have found above between moulds of group II and of group I for NaDDC.

With the propyl and dibutyl compounds the concentration of the 1 : 1 complexes is still less than with the diethyl compound (21) because of the lower solubility of the 1 : 2 complexes (15). Even *G.cingulata* does not show a first zone of inhibition now. *Bac. subtilis* however, appears to be more sensitive: on glucose agar with 5 p.p.m. of copper sulphate one finds inhibition with 1, 2, 5 and 10 p.p.m. dipropyl derivative and incomplete inhibition with 1 and 2 p.p.m. dibutyl derivative.

The free diethyl compound is as toxic as the free dimethyl compound (21), *i.e.* it acts at *ca.* 50 p.p.m. The free dipropyl compound acts at *ca.* 500 p.p.m. whilst the dibutyl compound is not yet inhibitory at this concentration. The same was found for *Bac.subtilis*. As we know that the chelating ability of the dithiocarbamates increases from methyl to butyl (14) it seems unlikely that the action of the free dithiocarbamates is due to chelation, unless permeability of these free compounds differs considerably.

We know only few compounds with an 1 : 2 complex having the "high" solubility of the dimethyl or diethyl compound. Na-cyclopentamethylenedithiocarbamate shows an activity of the 1 : 1 complex which is somewhat less than that of the diethyl compound; also the solubility of its 1 : 2 complex with Cu was somewhat less than that of the diethyl compound (15). The activity of the 1 : 2 complexes seems to be determined rather by solubility than by structure. But another factor is also involved, since bis (hydroxyethyl) dithiocarbamate is not toxic, yet it can be expected to have a higher solubility than the dialkyl derivatives. It has been found that the 1 : 2 complex with Cu rapidly decomposes with formation of a complex of univalent copper. So the picture becomes fairly complicated although up till now all facts can be related to the chemical and physicochemical properties of the dithiocarbamates.

The conclusion from these investigations seems to be that in general the activity of the dialkyldithiocarbamates at low concentrations is determined by the solubility of the 1 : 2 complex with Cu and by the sensitivity of the mould.

Comparison of the action of 8-hydroxyquinoline and pyridine-2-thiol-N-oxide with that of the dithiocarbamates:

We have already mentioned that the action of oxine can also be antagonized by histidine and other imidazole derivatives. This can also be affected by EDTA and dibutyldithiocarbamate, antagonism never going beyond 5 p.p.m. of oxine (19). This suggested that oxine has the same mode of action as NaDDC. ALBERT (1,3) has shown that the bactericidal action of oxine is effected by the 1 : 2 complex which is supposed to penetrate rapidly into the cells and to be transformed there into the actual toxic agent: the 1 : 1 complex. The latter is supposed to penetrate slowly and thus to act only when high concentrations are present in the outside medium. BLOCK (5) confirmed this for fungi.

We carried out the same experiments with oxine and $CuSO_4$ as we did with NaDDC (19); equally the amounts of 1 : 1 complex and 1 : 2 complex were calculated (Table 6 and 7).

The inhibition picture of *A.niger* is drawn in these tables. It appeared that moulds of all three groups showed the same picture now and this was the picture which we found for *G.cingulata* and NaDDC; there was no inversion growth. Accordingly $Cuox_2$ appeared inhibitory to moulds of all three groups (Table 8). Clearly the figures in Tables 6 and 7 show that for *A.niger* there is no correlation between inhibition and amount of Cuox in the medium unless perhaps at high Cu concentrations. The amounts of $Cuox_2$ show, however, a correlation with inhibition, 0.03 p.p.m. being about the inhibitory concentration. This is just about the value which we find inhibitory when $Cuox_2$ is added as such to the medium. Only when Cuox occurs in amounts $\geqslant 0.5$ p.p.m. (*e.g.*, in the combination of 0.5 p.p.m. oxine sulphate and 10 p.p.m. copper sulphate where only 0.02 p.p.m. of $Cuox_2$ is present) it seems to have a definite effect of its own.

Similar growth experiments have been described for bacteria by SORKIN, ROTH and ERLENMEYER (33). From their results it appeared that *Staphylococcus aureus* and *Mycobacterium tuberculosis* are inhi-

TABLE 6.

Amount of Cuox present (p.p.m.) in the medium.

p.p.m. copper sulphate added	p.p.m. oxine sulphate			
	0.05	0.1	0.2	0.5
0	0	0	0	0
0.03	0	0	0	0
0.1	0.03	0.04	0	0
0.3	0.05	0.08	0.1	0
1	0.05	0.1	0.2	0.3
3		0.1	0.2	0.5
10			0.2	0.5

-------- = Inhibition pattern of *Aspergillus niger*.

TABLE 7.

Amount of $Cuox_2$ present (p.p.m.) in the medium.

p.p.m. copper sulphate added	p.p.m. oxine sulphate			
	0.05	0.1	0.2	0.5
0	0	0	0	0
0.03	0.04	0.04	0.04	0.04
0.1	0.02	0.06	0.14	0.14
0.3	0.007	0.03	0.09	0.4
1	0.002	0.008	0.03	0.2
3		0.003	0.01	0.07
10			0.004	0.02

----- = Inhibition pattern of *Aspergillus niger*.

TABLE 8.

Solubility and inhibitory concentration of various complexes.

	solubility in p.p.m.	min. inh. conc. in p.p.m.		
		G. cingulata	*A. niger*	*F. oxysporum*
$Cuox_2$	1	0.02-0.05	0.1	0.2
$CuPTO_2$	0.2	0.1	0.2	
$CuDDC_2$	0.01	0.05	no inh.	no inh.
Cu diethyldithiocarbamate	0.002	partial inh.	no inh.	no inh.

bited by $Cuox_2$ but the activity increased by the addition of more Cu. Histidine must have been present in these media and since it also combines with Cu, it seems difficult to evaluate the amounts of Cuox and $Cuox_2$ actually present.

In Table 8 some figures are also given for pyridine-2-thiol-N-oxide (PTO) a compound that shows the same response as oxine in the presence of various amounts of Cu (19). ALBERT (3) reported that the mode of action of PTO on bacteria is comparable to that of oxine.

Oxine and PTO, like NaDDC, can be antagonized by stronger Cu binders as *e.g.* dibutyl dithiocarbamate. EDTA and imidazole derivatives are also antagonistic and will act as exposed above for NaDDC. Antagonistic activity never surpasses the barrier of 5 p.p.m. of oxine or PTO, and we suppose that from here onwards the free compound is inhibitory. α-Keto acids have no antagonistic activity.

Thus there is a very close correlation in the mode of action of dialkyldithiocarbamates, oxine and PTO.

Theories on the site of action of NaDDC:

It will be clear that since NaDDC can act in three different ways (1 : 1 complex, 1 : 2 complex and dithiocarbamate ion) each of these forms could have a different mode of action. We think it, however, most likely at present that the two complexes both act within the cells as the 1 : 1 complex, much in the same way as ALBERT suggests for the complexes of oxine: The lipophilic 1 : 2 compound penetrates better than the hydrophilic 1 : 1 compound, but the latter is the actual toxic agent which combines with an essential enzyme. The 1 : 2 complex then must be transformed into the 1 : 1 complex within the cells. The mode of transformation is as yet unknown. The dithiocarbamate ion acts probably on a quite different enzyme and there is reason to believe that the complexes act more fungicidally whereas the ion acts more fungistatically, but conclusive experiments are still lacking.

From Warburg experiments described by VAN DER KERK and KLÖPPING (23) it follows that O_2 consumption of *A.niger* in malt is left undisturbed by the free dithiocarbamate ion up to about 500 p.p.m. GOKSØYR (9) found that O_2 consumption of *Saccharomyces cerevisiae* with glucose as a substrate was only slightly inhibited by concentrations of 1 : 1 complex of NaDDC which did inhibit growth. With acetate as a substrate, inhibition of respiration was much decreased. Moreover, acetate incorporation was strongly inhibited by the 1 : 1 complex. The acetate-activating properties of a yeast extract were inhibited by *ca.* 20 p.p.m. TMTD, but not by NaDDC with or without addition of Zn (5). GOKSØYR (10) found, moreover, that in cell-free homogenates, but not in intact cells 2-20 p.p.m. NaDDC caused strong inhibition of succinic acid dehydrogenase, especially in the presence of *ca.* 15 p.p.m. zinc sulphate (9). The Cu complexes were inactive. SISLER and COX (23) described that the anaerobic fermentation of glucose by *Saccharomyces cerevisiae* was strongly inhibited by 20 p.p.m. of TMTD and this was due to inhibition of the oxidation of glyceraldehyde-3-phosphate.

Some other enzyme complexes are known to be inhibited by low concentration of NaDDC or TETD (tetraethylthiuram disulphide, the oxidation product of sodium diethyldithiocarbamate). Thus KEILIN and HARTREE (22) reported that *ca.* 1-3.5 p.p.m. TETD caused strong inhibition of the succinic dehydrogenase of heart muscle. We found that CuDDC, but not $CuDDC_2$, gave rise to some accumulation of pyruvic acid by *A.niger*, whereas the dithiocarbamate ion caused much stronger accumulation of this metabolic product (20). Acetaldehyd oxidase of liver homogenate appeared to be inhibited by 0.06 p.p.m. TETD (KJELDJAARD (24)) and by 1 p.p.m. TETD (GRAHAM (11)). NYGAARD and SUMNER (26) reported that 1 p.p.m. TETD inhibited D-glyceraldehyde 3-phosphate dehydrogenase from muscle.

These reports seem sometimes contradictory and the role played by metals is far from clear. Yet, when one considers the enzyme systems which can be inhibited by low concentration of NaDDC, TMTD or TETD, it is striking that with the exception of glyceraldehyde dehydrogenase all are known to contain an essential dithiol group; the dithiol compound dihydrolipoic acid functions in pyruvate oxidase (12), in the acetate-activating system of animal tissues (27) and yeast (28); in addition dithiol groups act in acetaldehyde oxidases (13) and their presence in succinic acid dehydrogenase was made acceptable (30). Arsenite is known to act on all these enzyme systems (12, 13, 18, 30) and this is due to interference with the dithiol group. This means that there is a parallel between enzyme inhibition by arsenite and dithiocarbamate, though arsenite is not growth-inhibiting in concentrations which produce strong effects on the enzymes mentioned.

Thus there seems to be much reason to suggest that NaDDC acts in general on enzymes which contain dihydrolipoic acid as coenzyme.

We know that an analogue of dihydrolipoic acid causes growth inhibition of bacteria and a yeast (6). The compound appeared to inhibit pyruvate oxidase and phosphotransacetylase (4). Growth inhibition by the analogue is thought to be due to interference with one of the functional forms of lipoic acid concerned in acetyl transfer, and in our opinion it may well be that growth inhibition by NaDDC is due to interference with the same system.

Little is known about the role which copper plays in the inhibition by NaDDC of the enzymes mentioned, but it seems acceptable that dithiols react rather with the 1 : 1 complex than with the dithiocarbamate ion.

Knowledge about the action of oxine or PTO and their metal complexes on enzyme systems with essential dithiol groups is very scanty. We have, however, found that pyruvic acid accumulation can be effected by oxine and by its 1 : 1 complex with Cu, in the same way as described above for NaDDC.

Summary.

In the action of sodium dimethyldithiocarbamate on moulds and bacteria the formation of its 1 : 1 and 1 : 2 complexes with Cu^{++} from the medium plays a decisive role. These Cu complexes on the whole have a higher toxicity than NaDDC itself. The response pattern varies, however, according to the sensitivity of the organism used.

The activity of higher homologues of NaDDC is determined by the solubility of their 1 : 2 complexes.

The mode of action of the dialkyldithiocarbamates is closely comparable with that of oxine and pyridine-2-thiol-N-oxide.

It is suggested that dithiocarbamates act by interference with some function of lipoic acid.

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A COMPARATIVE STUDY OF THE GENERA *GONYTRICHUM* AND *BISPOROMYCES*

by

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(Received July 27, 1959).

INTRODUCTION.

During a study of the micro-organisms in soil from the Frankenwald Experimental Station near Johannesburg, South Africa, a fungus was isolated that proved to be difficult to identify. A culture was sent to the Centraalbureau voor Schimmelcultures in Baarn (Netherlands) where it was concluded that it was most probably a new species in the genus *Bisporomyces* van Beyma. Some time after this the author found an illustrated description of *Gonytrichum macrocladum* (Sacc.) Hughes and concluded that the fungus under discussion was most probably identifiable with that organism. During a recent visit to Baarn a pure culture of *Gonytrichum macrocladum* was compared with the two species of *Bisporomyces* known at present (VAN BEYMA, 1940; MANGENOT, 1953). The results of this study show the two genera to have one feature in common that has not yet been described in *Gonytrichum*. The subsequent study of some herbarium material has confirmed this.

OBSERVATIONS.

The organism that started this investigation is illustrated in fig. 1. Two features should be noticed, firstly the branching of the conidiophore which takes place in a very characteristic way. Only one side branch develops just underneath each septum. This branch curves around the main axis, sometimes with a side branch curving the opposite way, and the sterigmata arise from these curving branches. This method of branching is regarded by HUGHES (1951) as the main diagnostic feature of *Gonytrichum*. The second point of interest

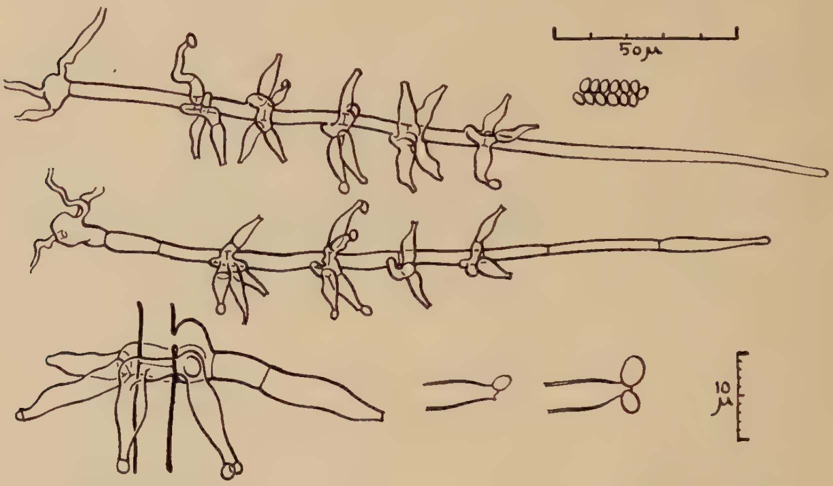

Fig. 1. Culture 225, isolated from Frankenwald soil. For further explanation see text.

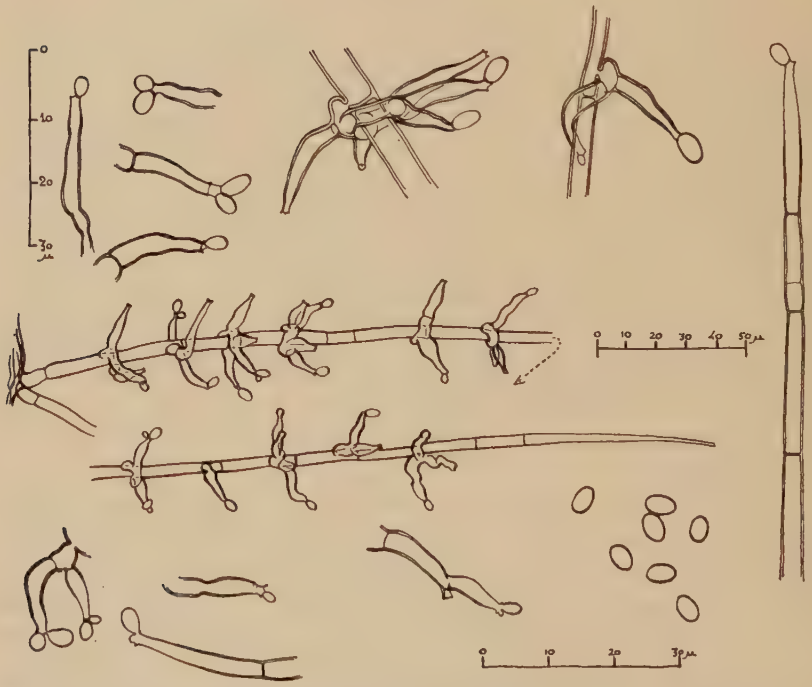

Fig. 2. *Gonytrichum macrocladum*, culture isolated by Atkinson. For further explanation see text.

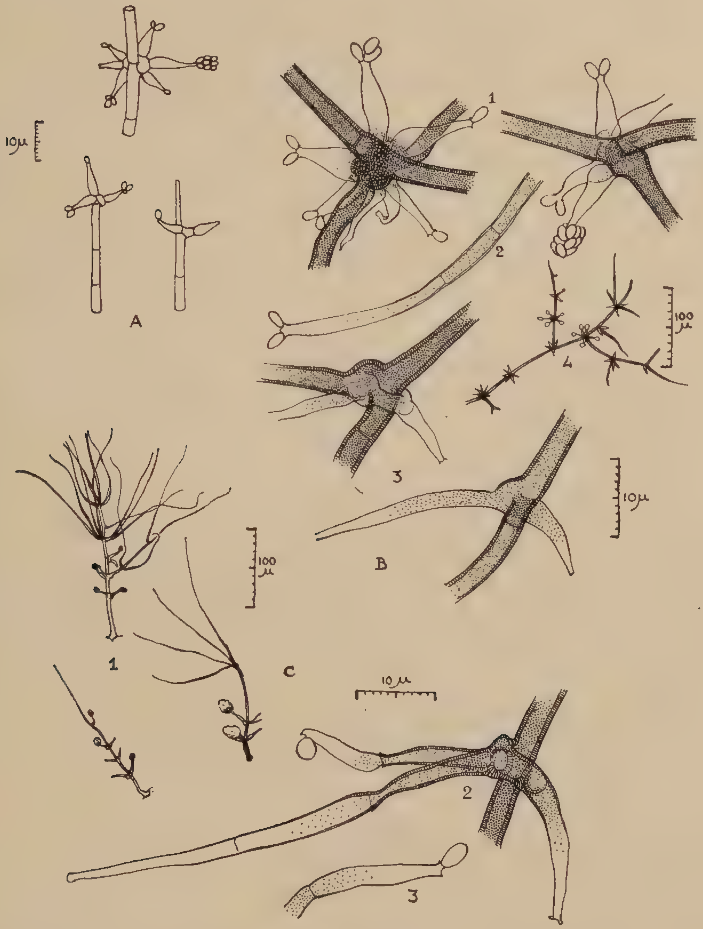

Fig. 3. A. *Gonytrichum caesium*, phialides as illustrated by HUGHES. B. *G. caesium* – Herb. I.M.I. 19265: 1, phialides; 2, tip of seta; 3, method of branching; 4, whole conidiophore. C. *G. macrocladum* – Herb. I.M.I. 1473: 1, whole conidiophores; 2, method of branching; 3, phialide.

is the way in which conidia are produced on the phialides. Each phialide has a double top, surrounded by a small single collar, and two conidia can be produced at the same time. This is the main diagnostic feature of the genus *Bisporomyces* as erected by VAN BEYMA. In HUGHES' description of the two species of *Gonytrichum* this second feature is not mentioned, although his illustration of *G. caesium* indicates that it might be present -- see fig. 3A.

A comparison of figs. 1 and 2 shows that the fungi illustrated there are identical. This second illustration has been made from a culture isolated by ATKINSON, seen by HUGHES and identified as *G. macrocladum*. The double top of the phialides is evident. Further evidence is given in fig. 3: A shows a detail of HUGHES' illustration of *G. caesium* Nees, the conidial form of *Melanopsamella inaequalis* (Grove) v. Höhnel; B shows details from the same material as from which HUGHES prepared his illustration (Herb. I.M.I. 19265); C shows details of *G. macrocladum*, again from the same material as illustrated by HUGHES (Herb. I.M.I. 1473). In old and somewhat degenerated cultures the proliferation of phialides occurs in *Gonytrichum* in the same way as it does in *Bisporomyces*: compare figs.4 and 5. It is interesting to note that in *Gonytrichum* the tips of the main axis and of the setae may become sterigmatic (fig. 2, fig. 3 B).

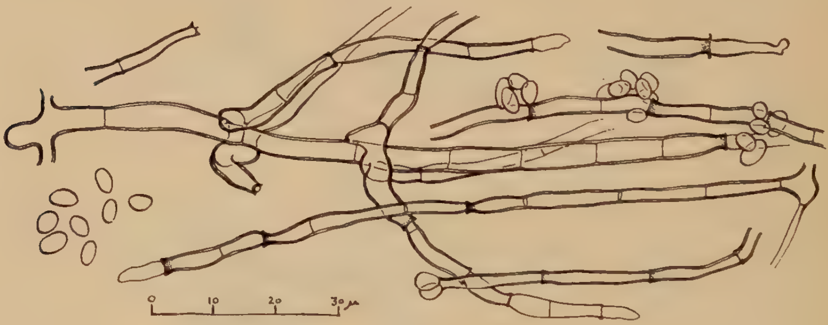

Fig. 4. Culture 225 – *Gonytrichum macrocladum*, degenerated, showing proliferating phialides.

It is shown above that the biphialide, the diagnostic feature of the genus *Bisporomyces*, is equally characteristic for the genus *Gonytrichum*. The typical pattern of branching in *Gonytrichum* is entirely absent in *Bisporomyces*.

In a recent article HUGHES (1958) reduced the name *Bisporomyces* to a synonym of *Chloridium* after studying type material. In fig. 6 an illustration of the type material of *Chloridium* is given (*Chloridium viride* Link: Botanical Garden and Museum, Berlin, Germany). Although it was not possible to see two conidia being produced from one phialide, all details observed indicate that this is most probably the case. The conidial apparatus does not show branching.

Chlamydospores are present. The observations leave little doubt that HUGHES' conclusion is correct.

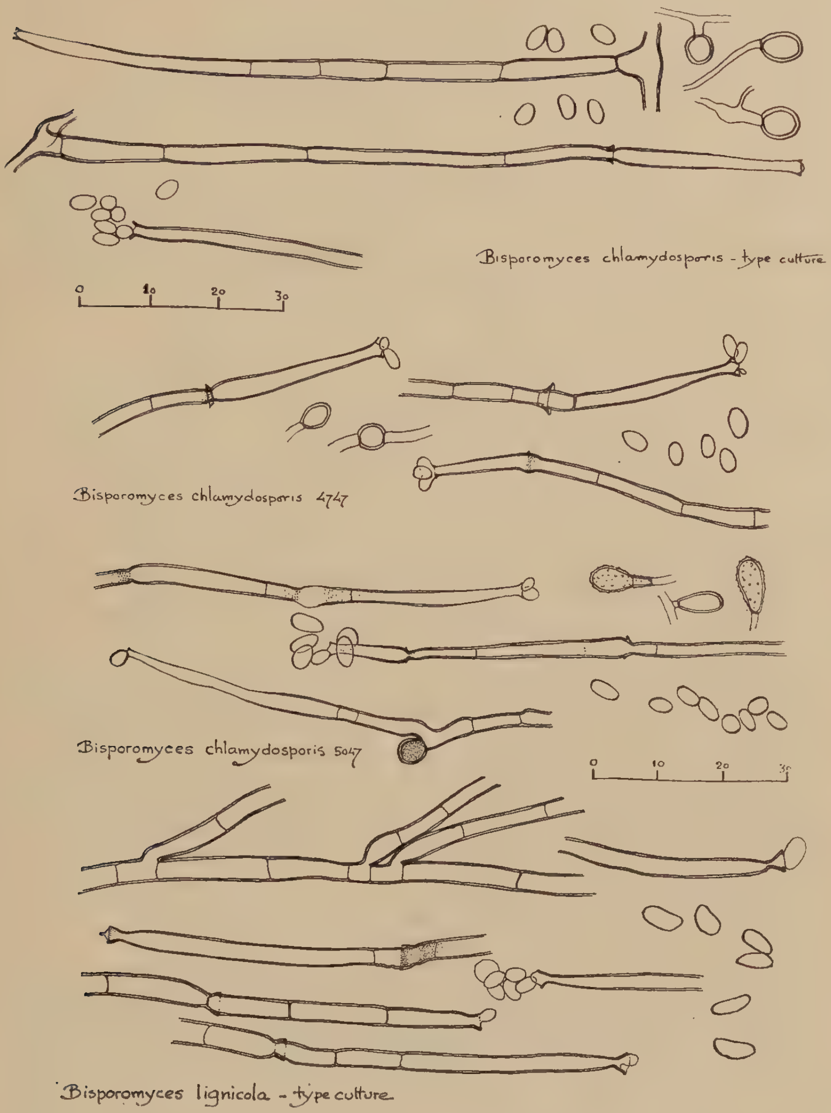
Bisporomyces chlamydosporis - type culture
0 10 20 30
Bisporomyces chlamydosporis 4747
Bisporomyces chlamydosporis 5047
0 10 20 30
Bisporomyces lignicola - type culture

Fig. 5. *Bisporomyces*, both species.

CONCLUSION.

Although the genus *Gonytrichum* appears to be closely related to *Chloridium* (*Bisporomyces*) it can be separated from this genus on account of its method of branching. The eventual discovery of an

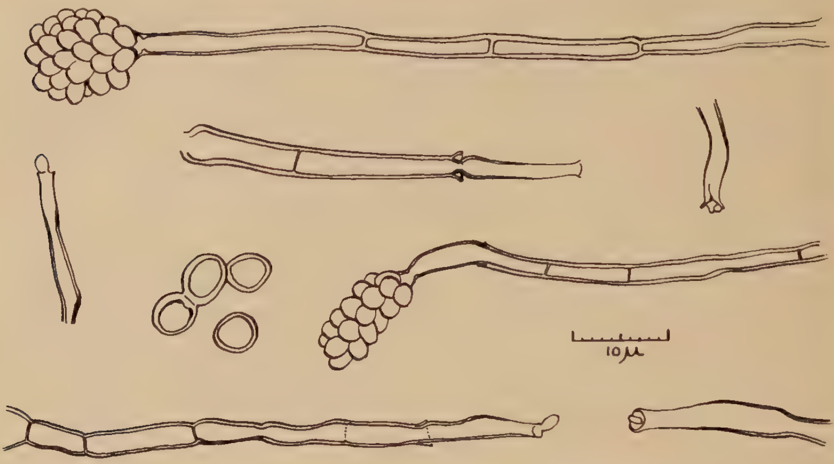

Fig. 6. *Chloridium viride,* drawn from type material.

ascus form connected with *Chloridium* might throw light on the true natural relationships between the two genera.

Summary.

A comparative study of the genera *Gonytrichum* and *Bisporomyces* indicates a close relationship between them as in both genera conidia are produced in the same rather exceptional way. The genera can be kept separate as *Gonytrichum* shows a very characteristic method of branching whereas *Bisporomyces* has unbranched conidiophores. The recent finding of Hughes that *Bisporomyces* is a synonym of *Chloridium* appears to be correct.

Acknowledgements.

The author is very grateful to the staff of the Centraalbureau voor Schimmelcultures, Baarn, Netherlands, for the opportunity given to study the pure cultures mentioned. He wishes to thank the Director of the Botanical Garden and Museum, Berlin, for lending the type specimen of *Chloridium viride,* and the Director of the Commonwealth Mycological Institute, Kew, for supplying duplicate herbarium specimens of the two species of *Gonytrichum.* He is indebted to Miss F. D. Hancock, M. Sc., for checking the manuscript.

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METHODE RAPIDE ET SIMPLE POUR DETERMINER LE DEGRE DE FERMENTATION DU RAFFINOSE PAR LES LEVURES

par

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(Reçu le 11 Mai 1959).

La fermentation de ce trisaccharide est importante dans la taxonomie des levures. Certaines d'entre elles fermentent complètement ce sucre, donc, les trois monosaccharides (fructose, glucose et galactose) qui entrent dans sa molécule et les deux disaccharides (saccharose et mélibiose) issus de l'union du glucose avec le fructose d'une part, et avec le galactose de l'autre. D'autres fermentent les deux tiers de la molécule de raffinose (le fructose et le glucose); elles sont capables de scinder le trisaccharide en ses trois monosaccharides composants, mais elles ne synthétisent point de galactozymase; elles sont donc incapables de fermenter le galactose. D'autres enfin, ne fermentent qu'un tiers de la molécule du raffinose (elles synthétisent du béta-fructosidase, qui hydrolise le saccharose, libérant le fructose, qui est fermenté par toutes les levures zymasiques; mais elles ne synthétisent pas l'alfa-galactosidase, enzyme responsable de l'hydrolise du mélibiose).

Afin de rendre plus clair notre exposé, nous présentons ci-dessous, d'une façon schématique, la composition du raffinose:

$$\overbrace{\text{FRUCTOSE}\qquad\rlap{$\underbrace{\phantom{\text{GLUCOSE}\qquad\qquad\text{GALACTOSE}}}_{\text{mélibiose}}$}\text{GLUCOSE}}^{\text{saccharose}}\qquad\qquad\text{GALACTOSE}$$

Parmis les méthodes imaginées pour l'étude de la fermentation du raffinose, les premières se basent sur la détermination quantitative du CO_2 dégagé durant la fermentation de ce trisaccharide et utilisent l'appareil de van Iterson-Kluyver, dont l'emploi est un peu compliqué et long. D'autres (Zimmermann, 1938) utilisent la titration des sucres résiduels à la fin de la fermentation. Henrici (1941) utilise la fermentation du mélibiose par la levure au lieu de celle du raffinose; mais cette méthode présente l'inconvénient de nécessiter quelquefois d'une adaptation prolongée de la levure, à moins de préparer le mélibiose à partir du raffinose, selon la méthode de Skinner et Bouthilet (1947). Wickerham (1943) emploie une méthode biologique avec *Saccharomyces carlsbergensis* (levure fermentant complètement le raffinose) comme témoin. Cette méthode ne permet pas de déterminer directement si le mélibiose formant partie de la molécule du raffinose est fermenté entièrement ou en partie. Pour y parvenir, il faut faire fermenter directement du mélibiose par la levure. Nous avions modifié cette méthode (Ramirez, 1953) en introduisant comme témoins, outre *S. carlsbergensis*, *S. cerevisiae* et *S. pastorianus*, suivant les cas. Nous arrivions ainsi à déterminer directement sur le raffinose quelle partie de sa molécule était fermentée par la levure étudiée. van Uden (1956) décrit une méthode chimique pour déterminer la fermentation du raffinose par les levures en se basant sur le fait suivant: le glucose, le galactose et le mélibiose sont réducteurs, tandis que le raffinose ne l'est point. Pourtant, on n'arrive pas à distinguer par cette méthode la fermentation partielle de la fermentation nulle du mélibiose de la molécule de raffinose. L'auteur pare à cet inconvénient en faisant fermenter directement du mélibiose par la levure étudiée.

Toutes ces méthodes sont plus ou moins longues; il leurs faut au moins une semaine pour aboutir à des résultats.

Notre nouvelle méthode est très simple, ne supposant pas un travail supplémentaire aux épreuves utilisées dans les déterminations taxonomiques des levures; elle est très rapide: les résultats sont fournis dans un délai de 24—48 heures; elle est économique, puisque nous employons dans les tests des quantités minimales de sucre. Par notre méthode nous ne faisons pas fermenter directement le raffinose dans des tubes de Einhorn, mais par contre nous employons la méthode auxanographique pour tester l'assimilation du raffinose et du mélibiose. Nous étudions en même temps, la fermentation du glucose et du galactose par la levure, dans des tubes de Einhorn, afin de déterminer si la levure étudiée est zymasique et galactozymasique.

Nous nous appuyons sur le fait suivant: le raffinose doit être hydrolisé en ses monosaccharides composants, avant d'être fermenté. Cette hydrolise est mise en évidence par la méthode auxanographique utilisée en taxonomie des levures pour l'étude de l'assimilation de ressources hydrocarbonées et azotées.

Nous pouvons postuler: si le raffinose est assimilé (croissance de la levure dans l'auxanogramme), la levure synthétise les enzymes qui hydrolisent, au moins en partie, ce trisaccharide; elle pourra donc fermenter les monosaccharides libérés dans l'hydrolise, si elle synthétise le complexe zymasique.

Par notre méthode, nous mettons en rapport la fermentation des monosaccharides formant partie de la molécule du raffinose avec les auxanogrammes de ce trisaccharide et du mélibiose.

Le tableau 1 montre les cas pouvant se présenter par l'emploi de notre méthode.

TABLEAU 1.

Détermination de la fermentation du raffinose pas les levures.

Auxanogramme		Fermentation		Partie de la molécule de raffinose fermentée (par déduction)
Raffinose	Mélibiose	Glucose	Galactose	
+	+	+	+	3/3
+	+	+	—	2/3
+	—	+	+	1/3
+	—	+	—	1/3
+	+	—	—	0
+	—	—	—	0
—	—	—	—	0

Sommaire.

L'auteur décrit une méthode rapide et simple pour la détermination de la fermentation du raffinose par les levures, en mettant en rapport l'hydrolise du trisaccharide (qui précède toujours la fermentation), par la méthode auxanographique, avec la fermentation du glucose et du galactose (monosaccharides formant partie de la molécule du raffinose). Les avantages de cette méthodes sont: 1) Rapidité et simplicité. 2) Economie des produits utilisés: le mélibiose et le raffinose sont employés dans les auxanogrammes en quantités minimales.

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THE WINE YEASTS OF THE CAPE

PART III. — THE FERMENTATIVE DISSIMILATION OF GLUCOSE BY *BRETTANOMYCES INTERMEDIUS* AND *BRETTANOMYCES CLAUSSENII* ¹)

by

J. P. VAN DER WALT and AMELIA E. VAN KERKEN

(Received June 5, 1959).

INTRODUCTION.

CLAUSSEN (1904) in his studies on the refermentation of English stock beers was the first to recognise the genus *Brettanomyces* as a taxonomic entity. It was not until 1940 with the appearance of a monograph on this genus by CUSTERS that more information on the metabolic activities of this interesting group of yeasts became available.

CUSTERS reported that the fermentative dissimilation of glucose by *Brett. claussenii* was anomalous; when conducting quantitative studies in a basal medium of yeast water he repeatedly found that the production of alcohol was greater, on a molecular basis, than that of the carbon dioxide evolved. For example, from 100 mM of glucose, 218 mM of ethanol and only 177 mM of carbon dioxide were recovered. This resulted in a carbon recovery of 102% despite the fact that glucose was utilised for cell synthesis. CUSTERS further showed that no volatile acid, acetaldehyde, butylene glycol or acetylmethylcarbinol was produced during the experiment. Under the same conditions *Sacch. cerevisiae* showed no deviation from the normal equimolar production of ethanol and carbon dioxide – 100 mM of glucose yielding 182 mM of ethanol and 176 mM of carbon dioxide (carbon recovery 90%). When the quantitative studies with *Brett. claussenii* were, however, conducted in dilute yeast water or dilute

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¹) Part I and II: Antonie van Leeuwenhoek 24, 239, 1958; 25, 145, 1959.

yeast autolysate, supplemented with 0.5% ammonium sulphate and 0.1% potassium dihydrogen phosphate, the normal equimolar yields of carbon dioxide and ethanol were obtained; *e.g.*, 100 mM of glucose was fermented to 177 mM of ethanol and 170 mM of carbon dioxide (carbon recovery 87.6%).

CUSTERS attributed the production of excess ethanol to the unique ability of *Brett. claussenii* to dissimilate some compound in yeast water, other than carbohydrate, to ethanol without the concomitant production of carbon dioxide. He did not attempt to identify this compound, however, and the problem has not been investigated further since.

The present authors (VAN DER WALT and VAN KERKEN, 1958, 1959) have shown that *Brett. intermedius* is largely responsible for the development of yeast hazes in finished wines in South Africa. As part of an investigation of the carbon source used for growth of this organism in dry white wines, it was considered to be of interest to repeat CUSTERS' work using this species. Contrary to expectations, it was found that *Brett. intermedius* did not produce any 'excess ethanol' in its fermentative dissimilation of glucose in yeast water, but yielded results similar to those obtained by CUSTERS for *Sacch. cerevisiae.* Further experiments were therefore carried out with *Brett. claussenii* using the same strain as used by CUSTERS. Again equimolar production of ethanol and carbon dioxide was observed. The validity of CUSTERS' results is therefore questioned.

EXPERIMENTAL.

Cultures: The following cultures were used:
(1) *Brettanomyces intermedius* CBS. No. 1943
(2) *Brettanomyces claussenii* CBS. No. 76.

Media: The following media were used:

(1) Yeast water 2% glucose;
(2) Diluted yeast water (1 : 3) + 0.5% $(NH_4)_2SO_4$ + 2% glucose;
(3) Dilute yeast autolysate (1 : 10) + 0.5% $(NH_4)_2SO_4$ + 2% glucose;
(4) Yeast water + 10% v/v yeast autolysate + 0.5% $(NH_4)_2SO_4$ + 2% glucose;
(5) Dilute yeast autolysate (1 : 10) + 1.5% Bacto casamino acids.

The yeast water was prepared as described by CUSTERS. The glucose was made up as an 8% solution which was sterilized separately and added to the sterile basal medium in the proportion of 1:3.

Fermentation: The apparatus and procedure were essentially the same as used by CUSTERS.

300 ml Basal medium was sterilized (10 lb, 15 min.) in a 500 ml cotton-plugged, round-bottomed flask with a standard ground joint. 100 ml Sterile glucose solution was added aseptically. A glass superstructure, sterilized separately, was fitted aseptically to the flask.

The superstructure consisted of a ground-glass joint into which had been sealed (a) a cotton-plugged tap funnel A which led to the bottom of the flask, (b) a side arm B, with tap, for the escape of gas, and (c) a second side arm C, which, like the funnel led to the bottom of the flask. All external exits of the superstructure were cotton-plugged. All ground-glass surfaces were greased with heat-sterilized silicone grease.

After connection of the flask and superstructure, the tap A was closed. B was connected to a bubbler with syrupy phosphoric acid. Sterile nitrogen, stripped of residual oxygen by scrubbing through a solution of chromous sulphate, was passed continuously through C and out at B. The flask was allowed to cool to room temperature when the gas flushing was stopped. Micro-aerobic conditions having been established, B and C were closed. The flask was then inoculated with yeast suspension through A and the suspension washed down with a few millilitres of sterile distilled water when the tap A was closed.

The yeast suspension was prepared by inoculating a vigorously growing slant culture of the yeast into 35 ml sterile malt extract with 1% Bacto yeast extract, in a cotton-plugged centrifuge bottle. After *circa* 5 days culturing at 30°C., the supernatant was discarded and the sediment washed twice aseptically with sterile water by centrifugation. The washed sediment was taken up in *circa* 15 ml sterile distilled water and transferred aseptically to the flask.

The equipment was set up at 30°C. and the bubbler connected to an absorption train for the gravimetric estimation of CO_2. The train consisted of (a) two U-tubes with anhydrone, (b) four tubes of soda-asbestos, (c) one tube with anhydrone, and (d) guard tubes of anhydrone and soda-asbestos. B was opened and fermentation was allowed to proceed.

After fermentation was completed (8-15 days), taps B and C were closed and the flask disconnected and shaken for 10 minutes. The flask was again connected and a wash bottle with concentrated sulphuric acid was introduced into the train just before the anhydrone tubes, to prevent excessive water vapour being introduced into the anhydrone, and CO_2-free air led through the flask *via* C and out at B to remove all residual CO_2 from the flask and anhydrone tubes for absorption in the soda-asbestos tubes.

The flask was disconnected. The contents was tested for contamination by plating. The contents was quantitatively transferred to a 500 ml volumetric flask and made up to volume for analysis.

Analytical methods:

Carbon dioxide – Determined by weighing the soda lime tubes.

Glucose – Estimated by the Hagedorn-Jensen method directly on the medium before and after fermentation.

Ethanol – Estimated according to the procedure of Braak (1928) by distillation and determination of the ethanol content of the distillate by oxidation with standardized bichromate according to the method of Semichon and Flanzy (1929) with *o*-phenanthroline as internal indicator (*cf.* Amerine and Joslyn, 1951).

RESULTS.

The results obtained with *Brett. intermedius* are given in Table 1–(a). Carbon recoveries are calculated as the sum of the weights of carbon in the carbon dioxide and ethanol recovered, expressed as percentage of the carbon in the glucose fermented. By available hydrogen is understood the number of hydrogen atoms liberated when one mole of a compound is completely dehydrogenated to carbon dioxide. The recovery of available hydrogen was calculated as hydrogen in the ethanol produced as percentage of that in the glucose fermented.

In all cases the molecular ratio of ethanol to carbon dioxide produced was unity within the limits of experimental error. In no case were more than two moles of ethanol produced per mole of glucose fermented. The recovery of carbon dioxide ranged from 81 to 100%. Recoveries of less than 100% are due, at least in part, to the utilisation of glucose for cell synthesis. Thus it was observed that cell growth was considerable in experiments 4 and 5 where recoveries of 81 and 86% were obtained. On the other hand, in experiments 2 and 3 where carbon recoveries were 100% and 96% respectively, little or no growth had taken place due to the fact that

TABLE 1.

Fermentative dissimilation of glucose by *Brett. intermedius* and *Brett. claussenii.*

Experiment No.	Basal medium [1]	Duration (days)	pH		Glucose fermented (g)	Fermentation products mM per 100 mM glucose fermented		Ratio mM ethanol / mM CO_2	Recovery of carbon (%)	Recovery of available hydrogen (%)
			After	Before		CO_2	Ethanol			
	a) Brettanomyces intermedius									
1	Yeast water	15	6.45	5.2	8.00	182	183	1.00	91.2	91.4
2	Yeast water	14	6.6	5.2	8.22	200	200	1.00	100.0	99.8
3	Dilute yeast water + 0.5% $(NH_4)_2SO_4$	14	6.1	3.85	7.93	189	192	1.02	95.7	95.8
4	Dilute yeast autolysate + 0.5% $(NH_4)_2SO_4$	8	5.8	4.4	8.42	163	162	0.99	81.3	81.9
5	Dilute yeast autolysate + 1.5% Bacto casamino acids	8	—	5.1	8.46	171	171	1.00	85.5	85.5
	b) Brettanomyces claussenii									
6	Yeast water	10	6.6	5.55	7.42	179	181	1.01	90.5	90.8
7	Yeast water + 10% v/v yeast autolysate + 0.5% $(NH_4)_2SO_4$	7	6.2	4.8	9.27	177	181	1.02	89.9	90.5
	c) Brettanomyces claussenii (CUSTERS' results)									
I	Yeast water	5	—	—	16.03	177	218	1.23	102.3	109.2
II	Yeast water	7	—	—	15.70	171	211	1.23	98.9	105.6

[1] All media contained 2% glucose.

the inoculum used was rather old and past its prime. Cell synthesis was generally much better in media to which additional nitrogen, either as ammonium sulphate or casamino acids, had been added. Dilute yeast water also gave less cell growth than did dilute yeast autolysate.

The recovery of the available hydrogen showed close agreement with the carbon recovery, as is to be expected with ethanol: carbon dioxide ratios of 1.

Reducing the organic nitrogen content of the medium, for example, by replacing part of the yeast water by ammonium sulphate or by increasing it by the addition of casamino acids and yeast autolysate, had no effect on the ratio of ethanol to carbon dioxide produced.

These results are at variance with those of CUSTERS for *Brett. claussenii*. He found ethanol : carbon dioxide ratios of 1.23 (see Table 1–(c)). Further experiments were therefore undertaken with *Brett. claussenii* to establish whether *Brett. intermedius* and *Brett. claussenii* differed in their fermentative dissimilation of glucose. These were done using the same strain as was used by CUSTERS. These results are shown in Table 1–(b). Again it was found that ethanol and carbon dioxide were produced in equimolar proportions. The deviations from unity of the ratios found in two experiments, *viz.*, 1.01 and 1.02 are within the limit of error of the determinations of the metabolites. Carbon and hydrogen recoveries were 90–91%.

DISCUSSION.

The results show that *Brett. intermedius* and *Brett. claussenii* show the same pattern of fermentative dissimilation of glucose in yeast water. This, however, differs significantly from that reported by CUSTERS for *Brett. claussenii* in the same medium. It is difficult to explain the difference in results in view of the fact that essentially the same media and technique, as well as the same strain of *Brett. claussenii*, were used as were employed by CUSTERS. The only difference was that our experiments were of longer duration.

It is difficult to conceive a fermentative dissimilation leading to the production of ethanol without the concomitant production of carbon dioxide as postulated by CUSTERS. Although NEUBERGER showed that yeasts can convert certain α-keto acids to acetaldehyde, the precursor of ethanol (*cf.* KLUYVER 1914) and KLUYVER (1914) showed that *Schizosaccharomyces pombe* can convert malic acid to ethanol, both these reactions produce equivalent amounts of carbon dioxide. The 'excess alcohol' could also not have arisen from α–amino acids by the Ehrlich mechanism as the oxidative de-amination leading to the formation of a keto acid is followed by decarboxylation with the production of carbon dioxide. Thus, the only possibility which remains is that ethanol and carbon dioxide were, in fact, produced in equimolar amounts from some compound in yeast water, but that the recovery of carbon dioxide was low because it was either assimilated or retained in the medium by some basic catabolite. As far as the first alternative is concerned, it has been shown by LIENER and BUCHANAN (1951) that yeast cells can obtain as much as 5% of their carbon requirements by the assimilation of carbon dioxide. It is unlikely, however, that this could account for CUSTERS' results because the excess of ethanol over carbon dioxide is too great. Moreover it is difficult to see why carbon dioxide assimilation should be found only in experiments carried out in yeast water and not in the other media employed by him.

It is therefore concluded that CUSTERS' claim that *Brett. claussenii* shows an abnormal fermentative dissimilation in the presence of yeast water cannot be substantiated. Both this species and *Brett. intermedius* show the same pattern of fermentative dissimilation as other yeasts.

Addendum.

Fermentative Dissimilation of Malic Acid by *Brett. intermedius*.

KLUYVER (1914) demonstrated that *Schizosacch. pombe* readily converted malic acid to ethanol and carbon dioxide – a property not shared by *Sacch. cerevisiae*. Since malic acid appears as a normal constituent in wine, the fermentative dissimilation of malic acid by *Brett. intermedius* was investigated.

The procedure adopted was the same as that used by KLUYVER (1914), *viz.*, the measurement in the van Iterson–Kluyver fermentometer of the amount of carbon dioxide evolved from 3% glucose and 3% glucose + 3% malic acid in yeast water. The results are recorded in Table 2.

The fact that the amount of carbon dioxide evolved in the medium containing 3% glucose + 3% malic acid is the same as that evolved

TABLE 2.

Fermentation of 3% glucose and 3% glucose + 3% malic acid by *Brett. intermedius* strain No. 18β.

Medium	Incubation period (days)	Vol. CO_2 evolved at NTP (ml)
Yeast water 3% glucose	5	7.7
Yeast water 3% glucose + 3% malic acid	11	7.7

from 3% glucose alone shows that *Brett. intermedius* cannot dissimilate malic acid under micro-aerobic conditions.

Summary.

The claims made by Custers in connection with the disproportionate yields of ethanol and carbon dioxide from the fermentative dissimilation of glucose in yeast water by *Brett. claussenii* were investigated for *Brett. intermedius*. All results obtained showed that *Brett. intermedius* produced the normal equimolar amounts of ethanol and carbon dioxide, irrespective of whether the studies were conducted in yeast water or in dilute yeast water supplemented with a suitable nitrogen source. Re-investigation of the fermentative dissimilation of glucose in yeast water by *Brett. claussenii* gave the same results as with *Brett. intermedius*.

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ENDOMYCES REESSII NOV. SPEC.

by

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During the course of investigations of the microflora associated with the cold-water retting of *Hibiscus cannabinus*, an extremely interesting sporogenous, hyaline mould was encountered from partially retted material from the Nelspruit area of the Eastern Transvaal. A closer study of the biochemical and morphological properties of the organism showed that it was possible to consider its classification in the genus *Endomyces* Reess as defined by LODDER and KREGER-VAN RIJ (1952) and by STELLING-DEKKER (1931). For many years since GUILLIERMOND (1909, 1912, 1928a, 1928b, 1935 and 1936) first enunciated his theories on the phylogenesis of the ascosporogenous yeasts, sporulating cultures of *Endomyces* species have not been available. The re-isolation and availability of such an actively sporulating culture was therefore considered to be of interest to mycologists and zymologists alike.

The following description is based on the standard methods of LODDER and KREGER-VAN RIJ (1952) and WICKERHAM (1951).

DESCRIPTION.

Growth in malt extract: After 3 days at 25° C., septate mycelium and arthrospores with rounded ends (2.5 – 6.3 μ in diameter) are formed. Reproduction solely by fission. A dull, wrinkled, white creeping pellicle and a sediment are formed.

Growth on malt agar: After 3 days at 25° C., septate mycelium and arthrospores with rounded ends (2.5 – 7.5 μ in diameter) are formed. Reproduction solely by fission. Sporangia with 1 – 2 sporangiospores are present.

After 1 month at 17° C., the culture is white, felted, spreading, flat, dull and slightly pulverulent. The margin is hirsute.

Slide cultures: A branched, septate mycelium is formed which breaks up into arthrospores.

Sporulation: Sexually mature mycelium forms protuberances with a somewhat granular content. More than one protuberance per cell may be formed (Figure 1 (a) (b) (c)). Protuberances are not always of the same size and do not develop at the same rate. However, no consistent differences which could lead to the distinction between male and female gametangia could be observed. After fusion, the protuberances increase in size. Two transverse septa are formed within the structure to form a sporangium (Figure 1 (c) (d) (e)). The sporangium increases in size with the formation of lipid globules. 1 – 2 Spores are delimited within the sporangium. These are prolate-ellipsoidal and contain a large lipid globule, $(5.0 - 6.3) \times (6.5 - 7.5)\ \mu$. The spores have a thick cell wall and are enveloped by a thin, creased membrane (Figure 1 (f) (g) (h) (i)).

Less frequently protuberances do not fuse and the terminal end may then enlarge while the contents becomes granular. By transverse septum formation at the base of the enlargement, a sporangium is formed in which a spore is delimited (Figure 1 (j) (k) (l) (m)).

The spores stain with the normal ascospore stains. No indication of somatogamous sexuality was observed.

Fermentation: Absent.

Carbon assimilation: Glucose, galactose, D-xylose, sorbitol, glycerol, ethanol, citric acid, succinic acid, malic acid and lactic acid are assimilated. Sorbose, saccharose, maltose, trehalose, cellobiose, lactose, melibiose, raffinose, melizitose, L- and D-arabinose, ribose, rhamnose, α-methyl glucoside, salicin, mannitol, erythritol, adonitol, dulcitol, m-inositol, potassium gluconate and tartrate are not assimilated.

Splitting of arbutin: Absent.

Assimilation of nitrate and nitrite: Absent.

Growth in the absence of an extraneous vitamin source: Absent.

DISCUSSION.

As the fusion of the protuberances which precedes sporangium formation in all likelihood constitutes a sexual process, these struc-

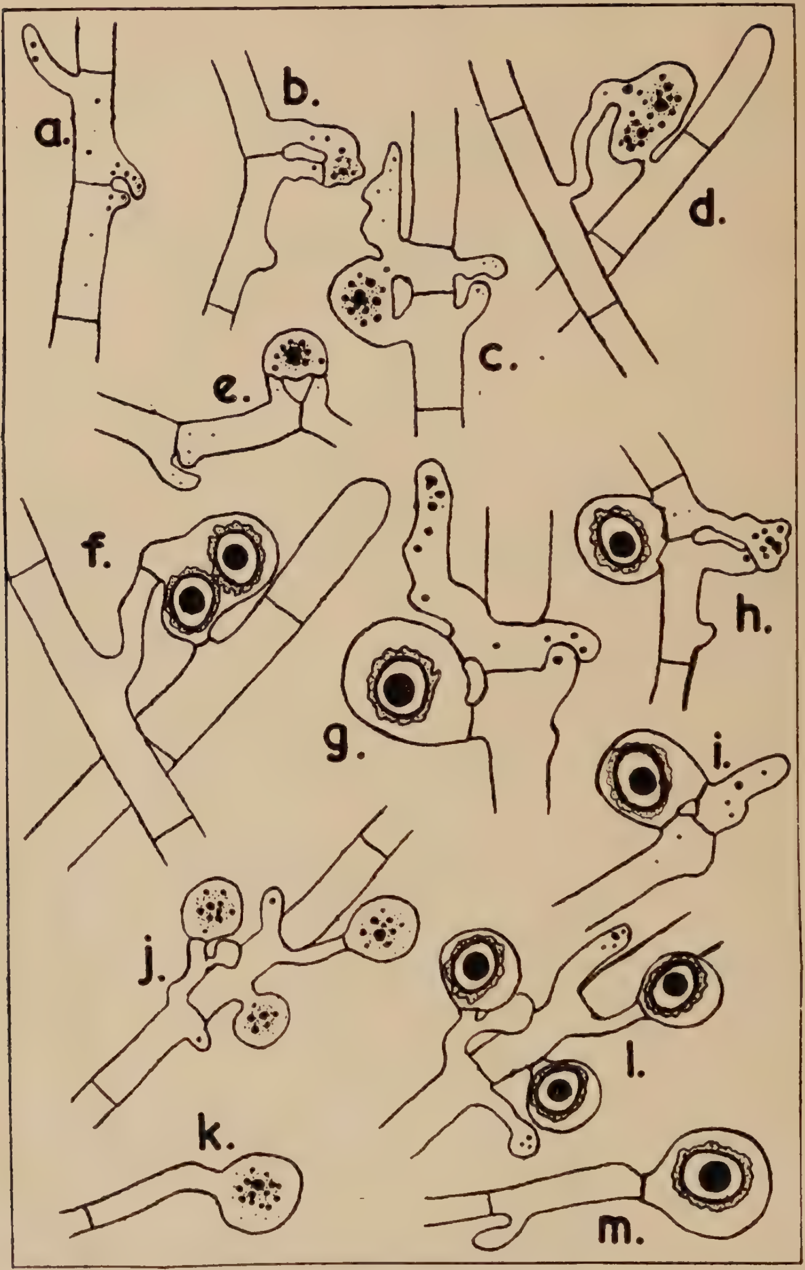

Fig. 1. *End. reessii.* Formation of gametangia, sporangia and sporangiospores (1000×, camera lucida drawings of structures as observed in a Ranvier-Winge chamber). a, b, c, d, e, j, and k after 3 days and f, g, h, i, l and m after 4 days, 25° C.

tures may be regarded as gametangia. The organism is hyaline; its sporangiospores stain with the normal ascospore stains; it forms a true mycelium and arthrospores and propagates solely by fission. In view of these properties, it seems appropriate to assign it to the genus *Endomyces* as defined by LODDER and KREGER-VAN RIJ (1952) and STELLING-DEKKER (1931). Moreover its sporulation agrees very well as described by GUILLIERMOND (1912) for other species of the genus.

However, in describing its sporangia and spores as asci and ascospores, several suppositions are made. If the organism is an ascomycete, meiosis in the normal alteration of generations must be restricted to the sporangium. This implies that the gametangial nuclei arise mitotically in what is presumed to be haploid hyphae. A detailed cytological study of the plasmogamy and karyogamy prior to sporulation as well as a study of the ploidy of vegetative phase is therefore necessary to substantiate these assumptions.

STELLING-DEKKER (1931) recognized two *Endomyces* species, *viz.*, *End. decipiens* and *End. magnussi* although she could not verify actual sporulation. A comparison of her descriptions, however, establishes that our culture cannot be identified with either of these.

Mention must also be made of an organism, *End. lactis*, described by WINDISCH (1951). In old cultures of *Oospora lactis* he observed cell wall remnants attached to normal cells. He considered these to be ruptured and discharged asci as he had done previously in his claims for the ascospore formation in *Candida pulcherrima* and *Candida tropicalis* (WINDISCH, 1940). However, he neither stained nor demonstrated the heat resistance of the so-called ascospores. His cultures were re-examined by Miss W. CH. SLOOFF of the Yeast Division of the Centraal Bureau voor Schimmelcultures who could not substantiate his claims (Personal Communication). She did, however, observe numerous chlamydospores in the cultures. It therefore seems likely that the structures WINDISCH observed were in reality the same type of artifact as can be seen during the germination of the chlamydospores of *Cand. pulcherrima* (VAN DER WALT, 1952). Although our organism shows some resemblance, morphologically and biochemically, to *Oospora lactis*, there are no grounds to consider *Oospora lactis* as its imperfect form.

More recently, PELÁEZ COMPAMANES and RAMIREZ GÓMEZ (1956) described *Endomyces ovetensis*, which they had isolated from tanning liquor. This species formed eight or more spores per ascus and assimilates glucose, galactose and saccharose and cannot therefore be considered identical with the species isolated from the retted material.

The fungus described above may therefore be regarded as representing a new species. For the new species the name *Endomyces reessii* is proposed in honour of M. Reess who in 1870 erected the genus *Endomyces*.

Sporogenous cultures of *Endomyces* species have been unavailable for many years since Guilliermond (1909) elaborated his hypothesis of the phylogenesis of the ascosporogenous yeasts from *Eremascus fertilis*. The availability of such cultures now permits mycologists and zymologists alike to re-evaluate his theory by more modern techniques.

According to Guilliermond's theories which have greatly influenced the taxonomy of the sporogenous yeasts during the past fifty years, the genus *Endomyces* occupies an intermediate position between the ancestral form *Eremascus fertilis* which forms only hyphae and no arthrospores and the true yeasts. According to this hypothesis, *End. reessii* can be regarded as related through the hypothetical *Endomyces b* to the genera *Eremascus* and *Schizosaccharomyces*. Although Stelling-Dekker (1931) included the genus *Endomyces* in her monograph on the sporogenous yeasts, Lodder and Kreger-van Rij deleted this genus from the yeast domain. Like Stelling-Dekker, they did, however, include it together with the genus *Schizosaccharomyces* in the subfamily of the *Endomycetoideae*. In more recent years, however, other workers, particularly Wickerham (1951), have put forth very plausible arguments against considering *Eremascus fertilis* and other filamentous forms as possible yeast progenitors.

In his extensive study of the genus *Hansenula*, Wickerham (1951) noted that very marked evolutionary trends could be observed in the phylogenetic line. The most fundamental trend was the progressive change from the stabilized haplophase in the most primitive species to the stabilized diplophase in the most recently evolved species. A second trend was the gradual lessening of the dependence on an extraneous vitamin source as the species became more diploid. A third trend was the ability to evoke a gaseous fermentation of an increasing number of sugars. Of particular interest was the observation that the ability to form true hyphae became more apparent as the species became more diploid.

If these criteria are applied without reserve to *End. reessii*, it is clear that, since it forms hyphae, it cannot be regarded as haploid as GUILLIERMOND's theory requires. On the other hand, it is non-fermentative and auxo-heterothrophic, which are characteristics of haploid yeast species.

VAN DER WALT (1959), however, pointed out that in the yeast domain phylogenetic lines must exist in which the evolution of the sexual, biochemical and morphological properties are not synchronized as in the genus *Hansenula* and the proposed genus *Dekkeromyces* (WICKERHAM, 1951, 1957). It is therefore inadvisable to apply these criteria indiscriminately to other genera of the Endomycetales.

It is, however, evident that, if the concept of a phylogenetic line as the basis for the definition of a natural genus is accepted, then the filamentous forms such as *Eremascus fertilis* and *End. reessii* cannot be regarded as progenitors of the ascosporogenous yeasts.

Likewise, it is impossible to regard *Dipodascus uninucleatus* as a progenitor of the genus *Kluyveromyces* as was supposed by VAN DER WALT (1956a, b).

As more species of the genus *Endomyces* become available for comparative study, it will become apparent whether it is possible to establish a phylogenetic line in this genus, as has already proved possible for several other yeast genera.

Endomyces reessii nov. spec.

In extracto malti, mycelium verum et arthrosporae in terminis rotundatae, hyalinae, (2.5 – 6.3) μ in diameter. Post dies 3 pellicula alba, non nitida, sursum repens et sedimentum formantur.

In agar malti mycelium verum et arthrosporae in terminis rotundatae, hyalinae, (2.5 – 7.5) μ in diameter. Sporangia et sporangiosporae. Post unam mensem 17° C., cultura alba, plana, se sternens, implicata, non-nitida, paulum pulverulenta. Margine piloso. Mycelia septa ramosa dilabuntur in arthrosporas.

Copulatio gametangiorum conformationem sporangiorum praecedit. Aliquando transformatio gametangiorum in sporangia sine copulatione. Sporangiosporae, glabrae, ovoidiae, globulos olei continentes, (5.0 – 6.3) × (6.5 – 7.5) μ. Sporangiosporae in membrana tenuis et rugata, involutae; 1–2 in quoque sporangio.

Fermentatio nulla.

Glucosum, galactosum, D-xylosum, sorbitolum, glycerolum, ethanolum, acidum citricum, acidum succinicum, DL-acidum malicum et DL-acidum lacticum assimilantur.

Sorbosum, saccharosum, maltosum, trehalosum, cellobiosum, lactosum, melibiosum, raffinosum, melizitosum, L- et D-arabinosum, ribosum, rhamnosum, α-methyl-D-glucosidum, salicinum, mannitolum, erythritolum, adonitolum, dulcitolum, m-inositolum, kalium gluconicum et DL-acidum tartaricum non assimilantur. Kalium nitricum et natrium nitrosum non assimilantur.

Arbutinum non finditur.

Necessariae ad crescentiam sunt vitaminae externae.

Isolata ex partim soluta *Hibisco cannabino*.

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A SEARCH FOR THE *RUBENTSCHIKII* GROUP OF *DESULPHOVIBRIO*

by

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Baars (1930) demonstrated that species of sulphate-reducing bacteria able to utilize normal fatty acids, first described by Rubentschik (1928), differed from the ordinary *Desulphovibrio desulphuricans*. He named them *D. rubentschikii*, able to use acetate and butyrate as well as lactate, and *D. rubentschikii* var. *anomalus*, which used butyrate but not acetate. Since then there have been several reports of the isolation of these species but pure cultures are not available. *D. rubentschikii* would be expected to utilize raw organic materials more completely than *D. desulphuricans* for practical purposes such as the treatment of industrial wastes or the microbial production of H_2S. This report describes the examination of enrichment cultures of sulphate-reducing bacteria in acetate and butyrate media, and the failure to isolate anything other than pure strains of *D. desulphuricans* from them. The work formed part of a wider programme of research on the utilization of aliphatic acids for bacterial sulphate reduction; for reasons beyond the authors' control the programme will not now be completed.

Methods.

Media. Medium I, similar to that prescribed by Baars (1930) and quoted as medium A by Butlin, Adams and Thomas (1949), was used for all enrichment cultures; 0.1% (w/v) of the organic substrate (sodium acetate or butyrate in place of lactate) was added before inoculation. Where necessary the medium was supplemented with Difco yeast extract. A solid medium for the isolation of pure cultures was prepared by adding 1.5% agar washed in tap and then distilled water. Medium 2, a lactate-yeast extract medium (Medium C of BUTLIN, ADAMS and THOMAS, 1949) was used for the cultivation of pure strains of *D. desulphuricans*. Medium 3, a peptone-yeast extract-glucose medium (POSTGATE, 1951) was used for the cultivation of some non-sulphate reducing bacteria and a similar medium containing 1.5% agar was used to detect the presence of anaerobic contaminants in purified cultures of sulphate-reducing bacteria. Thioglycollic acid (0.01% w/v) was added to all media before inoculation to keep the redox potential at a level suitable for the growth of sulphate-reducing bacteria; $FeSO_4(NH_4)_2SO_4.6H_2O$ (0.05% w/v) was usually added to the medium as a sterile solution before inoculation. Growth of sulphate-reducing bacteria was indicated by blackening of the medium due to FeS formation. For samples of marine origin 2.5% NaCl was added to the media; all media were adjusted to pH 7.4 ± 0.2 before inoculation.

Cultivation. Enrichment cultures were grown in air-tight bottles (capacity 60, 30, 15 or 5 ml) with ground glass stoppers. These were completely filled with medium to ensure anaerobic conditions and incubated at 30°C. Pure cultures of sulphate-reducing bacteria were incubated in test tubes under oxygen-free N_2.

Purification of cultures. Single colonies were isolated from dilutions in agar media of enrichment cultures which had shown progressive changes in their volatile acid contents. Tubes containing acetate agar medium 1 plus 0.01% yeast extract, were inoculated as described by POSTGATE (1953). Isolated black colonies were macerated in a drop of basal medium without organic substrate and inoculated into a further series agar tubes. The black colonies were now almost entirely free of contaminant 'white' colonies; single black colonies were then transferred to liquid media containing acetate or lactate.

Non-sulphate reducing bacteria were isolated in a similar manner from agar tubes containing acetate, lactate or glucose + peptone as the source of organic material. Pure cultures were also obtained from single cells of a soil enrichment by micromanipulation.

Estimation of volatile acids. Changes in the volatile acid content of enrichment cultures were determined by titration in CO_2-free air after steam distillation and redistillation with $MgSO_4 + HgO$ (FRIEDEMANN, 1938). 80 ml of medium containing acetate or butyrate was inoculated; an air-tight bottle was completely filled and the remainder of the medium used for the estimation of the initial volatile acid content. Further estimations were made at intervals during growth. After each sample had been taken, the culture was transferred to a smaller bottle which was completely filled so that anaerobic conditions were maintained.

Estimation of H_2S. 5 ml of culture was pipetted immediately into excess iodine, acidified and back-titrated with standard $Na_2S_2O_3$.

RESULTS.

Sulphate-reduction using acetate. BAARS (1930) demonstrated that sulphate-reduction with acetate was accompanied by an equimolecular decrease in the acetic acid content:

$$CH_3COOH + H_2SO_4 \rightarrow 2CO_2 + H_2S + 2H_2O$$

Enrichment cultures were made from digested sewage sludge and from soil that had been enriched with calcium acetate and sulphate some weeks previously. Enrichments from marine and freshwater material were also obtained from Professor VAN NIEL's laboratory, Pacific Grove, California. Enrichment cultures in acetate medium containing 0.01% yeast extract showed progressive decreases in their volatile acid contents, and, in two samples tested, the amounts of H_2S produced were 95% and 72% of the theoretical yield (table 1).

TABLE 1.

Changes in acetic acid content during growth of enrichment cultures.

Culture		(Acetate expressed as μmole/ml)								
		Marine			Soil			Digested sludge		
		I	II	III	I	II	III	I	II	III
Time (weeks)										
0		26	14	14	16	13	24	24	20	19
1		16	11	—	—	—	—	—	—	—
2		—	5	—	—	—	3	—	5	—
3		13	—	—	7	5	—	18	1	—
4				—						—
5				3						—
6										3
H_2S produced (μmole/ml)	Theoretical			11						16
	Actual			10.5						11.5

Enrichment cultures containing 0.1% yeast extract grew well and H_2S was produced, but the acetic content did not alter; hence the yeast extract was being used preferentially as the organic carbon source. All subsequent subcultures were made in acetate media containing 0.01% yeast extract.

Acetate utilization was extremely slow in these cultures. For comparison Table 2 shows that, in an enrichment made from sewage in a lactate medium (medium 2), lactate at nearly double the concentration used for acetate disappeared in about a week.

TABLE 2.

Disappearance of lactate in an enrichment culture prepared from sewage.

The enrichment culture was grown in medium 2 and the lactate remaining obtained as the difference between the original lactate present and the amount of acetate observed analytically.

Time (days)	Lactate remaining (μmole/ml)
0	40
2*	20*
5	3.5
8	0

* interpolated from a second experiment.

Fifty-seven colonies of sulphate-reducing bacteria were isolated, six of which came from the marine enrichment. All grew in an agar medium containing acetate as the added carbon source, but failed to grow in liquid acetate media without agar unless the yeast extract content was increased to 0.1%. Additions of small amounts of yeast extract, agar or sodium bicarbonate did not enable growth to take place in liquid acetate media. All the colonies isolated grew well in lactate medium, they were checked for the presence of anaerobic contaminants and were free of them. Therefore the isolated strains behaved like *D. desulphuricans* and had probably grown as colonies in acetate media by utilizing impurities remaining in the agar. Four colonies grew in liquid acetate media, but proved to be mixed populations of sulphate-reducing bacteria and a facultatively anaerobic rod.

Sulphate-reduction using butyrate. A fresh water enrichment culture obtained at Professor VAN NIEL's laboratory was examined by similar procedures. The original enrichment cultures showed increases in their volatile acid content (Table 3) which would be consistent with the equation:

$$2CH_3CH_2CH_2COOH + H_2SO_4 \rightarrow 4CH_3COOH + H_2S$$

TABLE 3.

Changes in volatile acid content during growth of enrichment cultures in butyrate media.

(Butyrate expressed as µmole/ml).

Time (weeks)	Culture I	II	III
0	11	16	10
1	13	—	—
2	18	18	—
3	24	—	—
4		—	17
5		—	
6		26	
7		29	

Eighteen single colonies of sulphate-reducing bacteria were isolated from a butyrate enrichment; all grew in an agar medium containing butyrate but failed to grow in a liquid butyrate medium without agar. All grew in lactate medium and were therefore ordinary *D. desulphuricans.*

Syntrophy of D. desulphuricans with other bacteria. It was possible that sulphate reduction occurred in acetate or butyrate media because of syntrophic growth of *D. desulphuricans* with other bacteria capable of using acetate or butyrate but not of reducing sulphate.

In a attempt to isolate such syntrophs, fifty-four single colonies of non-sulphate reducing bacteria were isolated from acetate media or medium 3; six of these were obtained from single cells by micromanipulation. Mixtures were made of these non-sulphate reducing bacteria, singly and in groups, with pure cultures of sulphate-reducing bacteria isolated from the enrichments and maintained in lactate media; none reduced sulphate in acetate media. Similarly seventeen strains of non-sulphate reducing bacteria were isolated from the butyric enrichment in a butyrate medium or medium 3 and tested as a mixture with *D. desulphuricans* isolated from the enrichment.

The mixed population did not utilize butyrate for sulphate reduction.

As an alternative approach to this problem, selective inhibition of the sulphate-reducing bacteria was attempted. Ammonium monofluorophosphate selectively inhibits growth of *D. desulphuricans* by competition with sulphate (POSTGATE, 1952). If syntrophy were responsible for substrate oxidation, addition of monofluorophosphate to acetate or butyrate enrichments containing only small amounts of sulphate should prevent the sulphate-reducing bacteria from growing and thus produce an enriched population of anaerobes able to utilize the carbon source but not to reduce sulphate. On subculture into media containing sulphate but free of monofluorophosphate this population should be unable to reduce sulphate in the presence of the carbon sources, unless a culture of *D. desulphuricans* is added as well. Since monofluorophosphate is bacteriostatic and not bactericidal, the success of this approach depends on the inocula used being sufficiently small to prevent carry-over of the original sulphate-reducing bacteria into the media free of monofluorophosphate.

Enrichments in butyrate media without added sulphate (< 10 mμ mole SO_4''/ml) or added iron salt, but containing 0, 20, 200, 2000 mμ mole/ml $(NH_4)_2PO_3F$ were inoculated with 10^{-5} inoculum (*c* 10^3 bacteria transferred) of a washed cell suspension from a butyrate enrichment culture. Subcultures (10^{-5} inoculum) were made from these enrichments into butyrate media containing sulphate and an iron salt, and also into similar inoculated with *D. desulphuricans* isolated from the butyrate enrichment.

Sulphate-reduction did not occur in any of the bottles inoculated from the inhibited enrichments only, though other bacteria grew, hence the sulphate-reducing bacteria had successfully been diluted out. Though a grey precipitate was formed in the bottles inoculated with sulphate-reducing bacteria together with certain inhibited enrichments, sulphate reduction comparable to that obtained in the original enrichments was not observed.

An analogous test with the acetate-utilizing enrichment derived from sewage sludge gave a similar result: removal of sulphate-reducing bacteria with monofluorophosphate and their replacement with a pure strain of *D. desulphuricans* isolated from sewage sludge did not give a population able to reduce sulphate with acetate. The authors were unable to perform analogous tests on the other acetate enrichments.

Morphology. Enrichments and isolated colonies were frequently inspected. Pure cultures of sulphate-reducing bacteria all showed the typical comma-shape and progressive motility of *D. desulphuricans*. Such types were, in fact, only rarely seen in acetic or butyric enrichment cultures, though motile and non-motile rods were common. The pure cultures of non-sulphate-reducing bacteria were most often motile non-sporulating rods, though on several occasions motile bacteria having a vibrio form were observed.

DISCUSSION.

Seventy-five single colonies or cells of sulphate-reducing bacteria were isolated from soil, sewage, freshwater and marine enrichments in media containing acetate or butyrate as the organic carbon source. They all failed to grow in acetate or butyrate media in pure culture, yet grew readily in lactate medium, hence they were ordinary *Desulphovibrio desulphuricans*. Since many reports describing *D. rubentschikii* do not exclude the possibility that contaminant anaerobes were present, and POSTGATE (1953) showed that these are not always easy to detect; since acetate utilization during sulphate reduction is markedly slow; since, finally, no pure cultures of *D. rubentschikii* are available from recognized culture collections, the existence of this species should be viewed with reserve. The work reported here is inconclusive but suggests either (a) that sulphate-reducing bacteria of the *rubentschikii* group have special cultural requirements that are only satisfied by symbiotic non-sulphate-reducing bacteria or (b) that the phenomena of acetate or butyrate utilization result from the combined action of *D. desulphuricans* and acetate- or butyrate-utilizing contaminants which eluded us in this work.

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Summary.

Sulphate-reducing bacteria isolated from enrichment cultures able to utilize acetate or butyrate were exclusively *Desulphovibrio*

desulphuricans, unable to metabolize acetate or butyrate, and no evidence was obtained for the species *D. rubentschikii* reputedly able to utilize these substrates. It is probable that disappearance of these substrates in appropriate enrichments is due to syntrophy between *D. desulphuricans* and non-sulphate-reducing bacteria able to utilize acetate, but critical data supporting this view were not obtained.

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